From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

EXPLORING NOVEL ROLES OF METABOLIC ENZYMES MTHFD2 AND PFKFB3 IN CANCER GENOME STABILITY AND THEIR POTENTIAL AS ANTICANCER THERAPEUTIC TARGETS

Nadilly Y. Bonagas Villarreal

Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-203-6
Cover illustration by Sophia Ceder (http://ceder.graphics/)

Exploring novel roles of metabolic enzymes MTHFD2 and PFKFB3 in cancer genome stability and their potential as anticancer therapeutic targets

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Nadilly Y. Bonagas Villarreal

To be publicly defended at Air & Fire, Science for Life Laboratory, Tomtebodavägen 23a

Friday, 4th of June 2021 at 9:30 am

Principal Supervisor:
Professor Thomas Helleday
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Dr. Oliver Mortusewicz
Karolinska Institutet
Department of Oncology-Pathology

Dr. Nina Gustafsson
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Chris Lord
University of London
Institute of Cancer Research
Division of Breast Cancer Research

Examination Board:
Professor Sonia Lain
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Klas Wiman
Karolinska Institutet
Department of Oncology-Pathology

Professor Bo Stenerlöw
Uppsala University
Department of Immunology, Genetics and Pathology

“No digáis que, agotado su tesoro,
de asuntos falta, enmudeció la lira;
podrá no haber poetas; pero siempre
habrá poesía.
…
Mientras la ciencia a descubrir no alcance
las fuentes de la vida,
y en el mar o en el cielo haya un abismo
que al cálculo resista,
mientras la humanidad siempre avanzando
no sepa a dó camina,
mientras haya un misterio para el hombre,
¡habrá poesía!”

– Gustavo Adolfo Bécquer

POPULAR SCIENCE SUMMARY OF THE THESIS

According to the World Health Organization (WHO), cancer remains the second leading cause of deaths around the world. They estimate that about one in three people will develop cancer during their lives. Despite our best efforts to develop better treatments, our current ones still cause great discomfort and suffering to the patients and their families.

Approximately 90% of all cancer patients receive radio- and/or chemotherapy, which have considerable side effects. These harmful effects are usually caused by the treatment not being able to tell the difference between cancer and normal cells, which results in healthy tissue being affected as well. This highlights the need for more precise therapies with fewer side effects.

Previous studies show that certain proteins, such as PFKFB3 and MTHFD2, are commonly overproduced in cancer cells. While these proteins are normally involved in breaking down nutrients like sugars and folic acid to produce energy and cell components, we have discovered that in cancer they can have other functions like protecting the cell's DNA, i.e., the master code with instructions to how the cells should work and how to make new cells.

Under normal circumstances, MTHFD2 is almost non-existent in adult healthy tissue; it is present only in embryos before cells mature into specialized organs. In embryos, MTHFD2 is important for fast growth because it provides the building blocks for all the DNA being assembled in the new cells. These DNA building blocks, called nucleotides, are part of the reason pregnant women are encouraged to take more folic acid – the embryo's MTHFD2 requires folic acid to make nucleotides. Cancer cells, like embryonic cells, divide extremely fast and find it convenient to reactivate MTHFD2 in order to make more DNA building blocks to support their fast growth.

In the case of PFKFB3, we found that when DNA is damaged by radiotherapy for example, PFKFB3 helps recruit the repair proteins needed to make new DNA building blocks and fix the damage. If left unrepaired, the DNA damage would cause cell death, a mechanism put in place to prevent corrupted DNA code from being passed on to new cells. Therefore, tumors benefit from overproducing PFKFB3 to avoid death when their DNA is damaged.

Our group has collaborated with the pharmaceutical company Kancera to test their recently developed anti-PFKFB3 drug, while we have generated several drugs of our own that inhibit MTHFD2. These drugs kill the cancer cells which rely on PFKFB3 or MTHFD2, while largely sparing the healthy cells which do not have as much of these proteins. Our hope is that we can use these new drugs to treat many types of cancer more precisely, alone or in combination with radiotherapy and other drugs, to reduce the side effects for patients and even sensitize cancers which have become resistant to therapy.

ABSTRACT

Altered tumor metabolism has been described as early as the 1920s, but it was only in recent decades that proteomic and metabolomic studies revealed that the ways in which tumors rewire their nutrient and energy pathways are more diverse and have more implications for treatment outcome than previously thought. There is now a great interest in characterizing promising metabolic targets and identifying novel ways by which to exploit them for cancer treatment.

This thesis work is part of an ongoing effort to elucidate the molecular mechanisms behind metabolic cancer targets specifically at the interface of genome stability, their role in the pathogenesis of different tumor types and genetic contexts, and their suitability as drug targets for clinically relevant treatment strategies.

In **Paper I**, we present a new role for the glycolysis enzyme 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase 3 (PFKFB3) in homologous recombination (HR). We used gene silencing and pharmacological inhibitors to investigate the role of PFKFB3 in the response to DNA damage induced by ionizing radiation (IR). We found that PFKFB3 promotes the recruitment of DNA repair factors and supplies nucleotides for DNA synthesis through its interaction with ribonucleotide reductase (RNR). We also validated the antitumor preclinical potential of PFKFB3 inhibitor KAN0438757 and showed it specifically sensitized cancer cells to IR.

In **Paper II**, we solve the first crystal structure of human one-carbon metabolism enzyme methylenetetrahydrofolate dehydrogenase 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2) in complex with its cofactors and a weak inhibitor, LY345899. We developed biochemical activity and target engagement assays to evaluate the binding and inhibition of MTHFD2 by LY345899 in cancer cell models. With the newfound structural insights to determine key residues important for substrate and cofactor binding, we were able to undertake a structure-based drug discovery program targeting MTHFD2 detailed in **Paper III**.

Paper III expands on the groundwork laid out in Paper II to develop first-in-class, highly potent and cell active inhibitors of MTHFD2 (MTHFD2i). Again, using gene silencing techniques, we identified a novel role for MTHFD2 in genome maintenance, which we confirmed with our small molecule inhibitors. We show that MTHFD2i induce replication stress and apoptosis selectively in transformed cells as a result of impaired *de novo* thymidylate synthesis and genomic uracil misincorporation. We established an *in vivo* model of acute myeloid leukemia (AML) and showed that MTHFD2i significantly prolonged survival and outperformed the standard of care compound cytarabine (AraC), providing proof-of-concept for the translational potential of MTHFD2i as anticancer drugs.

In **Paper IV**, we further elaborate on the role of MTHFD2 in genome maintenance in response to DNA damage. We found that MTHFD2 accumulates and associates to chromatin upon DNA double strand breaks (DSBs) and promotes DNA repair through HR. Loss of MTHFD2

significantly impairs HR activity, with MTHFD2i specifically sensitizing cancer cells to PARP inhibitors *in vitro* and delaying tumor growth when combined with a PARP inhibitor *in vivo*.

Taken together, these studies showcase these two metabolic enzymes, PFKFB3 and MTHFD2, in a new light as novel DNA damage response (DDR) targets. Our findings provide compelling evidence to propose the intersection of cancer metabolism and genome stability as an untapped source of novel anticancer targets warranting more mechanistic and drug development efforts.

LIST OF SCIENTIFIC PAPERS

I. Gustafsson, N. M. S., Färnegårdh, K., **Bonagas, N.**, Huguet Ninou, A., Groth, P., Wiita, E., Jönsson, M., Hallberg, K., Lehto, J., Pennisi, R., Martinsson, J., Norström, C., Hollers, J., Schultz, J., Andersson, M., Markova, N., Marttila, P., Kim, B., Norin, M., Olin, T., and Helleday, T.
Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination.
Nat Commun (2018) **9**, 3872

II. Gustafsson, R., Jemth, A. S., Gustafsson, N.M.S., Färnegårdh, K., Loseva, O., Wiita, E., **Bonagas, N.**, Dahllund, L., Llona-Minguez, S., Häggblad, M., Henriksson, M., Andersson, Y., Homan, E., Helleday, T., and Stenmark P.
Crystal structure of the emerging cancer target MTHFD2 in complex with a substrate-based inhibitor.
Cancer Res (2017) **77**, 937-948

III. Gustafsson, N. M. S.*, **Bonagas, N.***, Henriksson, M.*, Wiita, E., Gustafsson, R., Marttila, P., Borhade, S., Vallin, K., Sarno, A., Svensson, R., Göktürk, C., Pham, T., Jemth, A. S., Loseva, O., Green, A. C., Cookson, V., Sandberg, L., Rasti, A., Unterlass, J. E., Haraldsson, M., Andersson, Y., Scaletti, E. R., Bengtsson, C., Paulin, C. B. J., Sanjiv, K., Abdurakhmanov, E., Pudelko, L., Kunz, B., Desroses, M., Iliev, P., Färnegårdh, K., Krämer, A., Garg, N., Michel, M., Häggblad Sahlberg, S., Jarvius, M., Kalderén, C., Bögedahl Jensen, A., Almlöf, I., Karsten, S., Zhang, S., Häggblad, M., Eriksson, A., Liu, J., Glinghammar, B., Nekhotiaeva, N., Klingegård, F., Koolmeister, T., Martens, U., Llona-Minguez, S., Moulson, R., Nordström, H., Parrow, V., Dahllund, L., Sjöberg, B., Vargas, I., Vo, D.D., Wannberg, J., Knapp, S., Krokan, H. E., Arvidsson, P. I., Scobie, M., Stenmark, P., Warpman Berglund, U., Homan, E., and Helleday, T.
Targeting MTHFD2 kills cancer via thymineless-induced replication stress.
Manuscript, submitted

IV. **Bonagas, N.**, Gustafsson, N. M. S., Albers, J., Rasti, A., Warpman Berglund, U., Mortusewicz, O., and Helleday, T.
Targeting MTHFD2 impairs homologous recombination and sensitizes cancer cells to PARP inhibitors.
Manuscript

* – Authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

Genes and proteins

53BP1	Tumor protein p53 binding protein 1
6PGD	6-phosphogluconate dehydrogenase
ACLY	ATP-citrate lyase
AKT	Protein kinase B (tyrosine kinase)
AMT	Aminomethyltransferase
ATM	Ataxia-telangiectasia mutated kinase
ATF4	Activating transcription factor 4
ATR	Ataxia- and Rad3-related kinase
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CAD	Carbamoyl-phosphate synthetase 2-aspartate transcarbamoylase-dihydroorotase
CDA	Cytidine deaminase
CDK1	Cyclin-dependent kinase 1
CDK4	Cyclin-dependent kinase 4
CHK1	Checkpoint kinase 1
CtIP	CTBP-interacting protein; Rb binding protein 8 (endonuclease)
DHFR	Dihydrofolate reductase
DHODH	Dihydroorotate dehydrogenase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dUTPase	Deoxyuridine triphosphate pyrophosphatase
EXO1	Exonuclease 1
FH	Fumarate hydratase
FOLH1	Folate hydrolase 1; PSMA1
FPGS	Folate polyglutamate synthetase
G6PD	Glucose-6-phosphate dehydrogenase
GARFT	Glycinamide ribonucleotide formyltransferase
GFP	Green fluorescent protein
GGH	Gamma-glutamyl hydrolase
GS	Glutamine synthetase
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1α
HK	Hexokinase
IDH	Isocitrate dehydrogenase
IMPDH	Inosine-5’-monophosphate dehydrogenase
KAP1	KRAB-associated protein-1; TRIM28; TIF1β
KRAS	Kirsten rat sarcoma oncogene homolog (GTPase)
Ku70	Thyroid autoantigen 70 kDa; XRCC6
Ku86	Thyroid autoantigen 80 kDa; XRCC5
MDC1	Mediator of DNA damage checkpoint 1
MRE11	Meiotic recombination 11
MRN	MRE11-RAD50-NBS1 complex
MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1
MTHFD1L	Methylenetetrahydrofolate dehydrogenase 1 like
MTHFD2	Methylenetetrahydrofolate dehydrogenase 2, methenyl-tetrahydrofolate cyclohydrolase

MTHFD2L	Methylenetetrahydrofolate dehydrogenase 2 like
MTHFR	Methylene tetrahydrofolate reductase
mTOR	Mammalian target of rapamycin
MYC	Myelocytomatosis homolog
NBS1	Nijmegen breakage syndrome 1
NDPK	Nucleoside diphosphate kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	Nuclear factor, erythroid 2 like 2
P27	Cyclin-Dependent Kinase Inhibitor 1B; Kip1
P53	Tumor protein p53; Tp53
PAICS	Phosphoribosylaminoimidazole carboxylase and phosphoribosyl-aminoimidazolesuccinocarboxamide synthase
PARP1	Poly(ADP-ribose) polymerase 1
PCFT	Proton-coupled folate transporter
PFK-1	6-phosphofructo-1-kinase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PGAM1	Phosphoglycerate mutase 1
PHGDH	Phosphoglycerate dehydrogenase
PI3K	Phosphoinositide-3-kinase
PKM2	Pyruvate kinase M2
PPAT	Phosphoribosyl pyrophosphate amidotransferase
PRPS2	Phosphoribosyl pyrophosphate synthetase 2
PTEN	Phosphatase and tensin homolog
RAD50	RAD50 double strand break repair protein
RAD51	RAD51 recombinase, RecA homolog
RAS	Rat sarcoma oncogene homolog
RFC	Reduced folate transporter
RNR	Ribonucleotide reductase
RPA	Replication protein A
RPA32	Replication protein A2; RPA2
RRM2	Ribonucleotide reductase regulatory subunit M2
SDH	Succinate dehydrogenase
SHMT1	Serine hydroxymethyltransferase 1
SHMT2	Serine hydroxymethyltransferase 2
SOD1	Superoxide dismutase 1
SUMO	Small ubiquitin-like modifier
TMPK	2’-deoxythymidine-5’-monophosphate kinase
TS	Thymidylate synthase; TYMS
UNG	Uracil DNA glycosylase
WEE1	WEE1 G2 checkpoint kinase
γH2AX	Histone H2A, variant X, phosphorylated at Ser139

Metabolites, drugs and compounds

2-DG	2-deoxyglucose
2-PG	2-phosphoglycerate
3-PG	3-phosphoglycerate
5-FU	5-fluorouracil
5-FUdR	5-fluorodeoxyuridine
5-meTHF	5-methyl tetrahydrofolate
5,10-meTHF	5,10-methylene tetrahydrofolate; CH_2-THF

Acetyl-CoA	Acetyl coenzyme A
AMP	Adenosine monophosphate
AraC	Cytosine arabinoside; cytarabine
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid
BrdU	5-bromo-2’-deoxyuridine
CldU	5-chloro-2'-deoxyuridine
CO_2	Carbon dioxide
dNTP	Deoxyribonucleotide triphosphate
dCTP	2’-deoxycytidine-5’-triphosphate
dTMP	2’-deoxythymidine-5’-monophosphate
dTTP	2’-deoxythymidine-5’-triphosphate
dUMP	2’-deoxyuridine-5’-monophosphate
dUTP	2’-deoxyuridine-5’-triphosphate
DMSO	Dimethyl sulfoxide
EdU	5-ethynyl-2′-deoxyuridine
F1,6BP	Fructose-1,6-bisphosphate
F2,6BP	Fructose-2,6-bisphosphate
F6P	Fructose-6-phosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HU	Hydroxyurea
IdU	5-iodo-2'-deoxyuridine
IMP	Inosine 5'-monophosphate
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OMP	Orotate monophosphate
PRPP	Phosphoribosyl pyrophosphate
SAM	S-adenosylmethionine
THF	Tetrahydrofolate
UMP	Uridine monophosphate

Other

ADME	Absorption, distribution, metabolism, and excretion
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CETSA	Cellular thermal shift assay
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9
DARTS	Drug affinity responsive target stability
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DR-GFP	Direct repeat green fluorescent protein
DSB	DNA double strand break
DSF	Differential scanning fluorimetry
ETC	Electron transport chain
HR	Homologous recombination
IR	Ionizing radiation

ITDRF	Isothermal dose-response fingerprint
mRNA	Messenger ribonucleic acid
NHEJ	Non-homologous end joining
PCR	Polymerase chain reaction
PPP	Pentose phosphate pathway
RNA	Ribonucleic acid
RNAi	Interfering ribonucleic acid
ROS	Reactive oxygen species
SAR	Structure-activity relationship
siRNA	Small interfering ribonucleic acid
SPR	Surface plasmon resonance
SSB	DNA single strand break
ssDNA	Single strand DNA
TCA cycle	Tricarboxylic acid cycle; Krebs cycle
Tm	Melting temperature
TSA	Thermal shift assay
UV	Ultraviolet
WHO	World Health Organization

1 INTRODUCTION

1.1 METABOLIC REPROGRAMMING IN CANCER

1.1.1 Oncogene-driven remodeling of metabolic and energetic pathways

The relationship between cellular energy metabolism and oncogenesis was first described almost 100 years ago in the 1920s by Otto Warburg and his colleagues (1). They discovered that tumor cells shift their glucose metabolism to favor increased rates of anaerobic glycolysis and lactose fermentation independently of oxygen availability, a phenomenon known today as the Warburg effect (1,2). At the time, many misinterpreted these observations to mean that cancer was caused by defective mitochondrial respiration and metabolism, including Warburg himself (3). This initial lack of understanding resulted in cancer metabolism being heavily criticized and largely dismissed for the greater part of the 20th century (2), and further overlooked with the rise of molecular oncology and cancer genetics in the late 1980s (4). The discovery of the first oncogenes and tumor suppressors held the promise of exposing the root cause of all neoplastic transformation, redefining the paradigm of cancer research for decades to follow; a view which held genetic alterations as the sole key driving force for oncogenesis, and relegated metabolic rewiring to a mere side-effect (5,6).

Today we understand that, despite increased glycolysis, mitochondrial metabolism is rather preserved by cancer cells and even indispensable for their growth and survival (7,8). The effects observed by Warburg are the likely result of deregulated glycolysis via hypoxia-inducible factor 1α (HIF-1α) modulation rather than defective mitochondria (9–12), effects which can exist to various degrees and concurrently with mitochondrial respiration in many cancer types (13,14). Over the past two decades, the development of highly performant biophysical and computational methods giving rise to the proteomics and metabolomics age have greatly rekindled the interest for studying the deregulation of metabolic pathways in cancer, with extensive data highlighting its importance for cancer development and progression (15–21). Nowadays considered a hallmark of cancer (22), the reprogramming of energy metabolism represents an important trait acquired by cancer cells to redirect cellular resources, including building blocks and energy supplies, to support the elevated levels of biosynthesis that accompany increased cell proliferation (23).

The connection between cancer genetic alterations and metabolic reprogramming remains a highly intricate one, with most oncogenes and tumor suppressors, including p53, MYC, RAS and AKT, having direct effects over major metabolic pathways, and vice-versa (**Figure 1**) (24–26). Most of these genetic and metabolic alterations induce deregulation of cell growth and division, providing the basis for anticancer chemotherapy. However, normal cells in the bone marrow and in the intestinal crypts, as well as other tissues, also undergo rapid proliferation, often at higher rates than cancer cells (27). This lack of cancer specificity has long represented the greatest limitation of chemotherapy, with severe myeloid and gastrointestinal toxicity often

being dose-limiting for these drugs (28). Since the rediscovery of tumor metabolism as a validated source of anticancer strategies, extraordinary efforts to uncover and target cancer-specific metabolic features have resulted in the approval and clinical success of new antimetabolic therapies in recent years, with several more in the development pipeline (29–32).

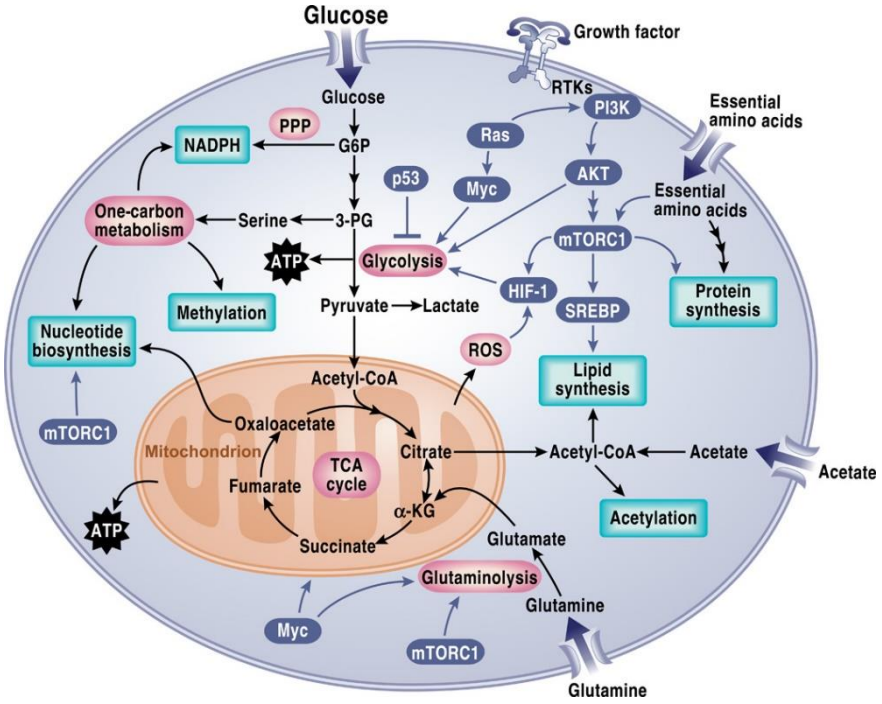

Figure 1. Oncogenic and tumor suppressor signaling regulating cancer metabolism. Oncogenes and tumor suppressors are shown in purple, key metabolic pathways are shown in pink, with the main biosynthetic areas labeled in blue. Reprinted from DeBerardinis & Chandel, 2016 (33), with permission from the American Association for the Advancement of Science (AAAS).

1.1.2 Metabolic alterations as a source of novel oncotargets

The link between cancer and altered cellular metabolism has been further validated by the discovery of cancer-associated mutations in metabolic enzyme genes (34). Thanks to advances in mass spectrometry and nuclear magnetic resonance methods used for the high-resolution profiling of low-weight metabolites (35–38), some of these metabolic gene mutations have been found to result in abnormal accumulation of metabolites at the root of cellular process deregulation and malignant transformation in cancer cells (25,34,39,40).

The best-known examples of metabolic oncotargets were identified using metabolic profiling of tumor cells which revealed abnormal accumulation of tricarboxylic acid (TCA) cycle intermediates as a result of loss- and gain-of-function mutations in genes encoding the metabolic enzymes succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH) (41–43). Deregulation of mitochondrial function characterized by TCA cycle defects has been associated with overproduction of reactive oxygen species (ROS), which is known to influence oncogenic signaling and tumor progression through the oxidative damage of proteins and nucleotides (44,45). Therefore, the metabolic pathways that lead to the

production of oncometabolites and the downstream signaling pathways that are activated by oncometabolites represent potential therapeutic targets, some of which are successfully exploited in the clinic today (**Figure 2**) (30,46,47).

Modern metabolomic approaches together with metabolic flux models using isotope tracers can provide direct pathophysiological insights into tumor metabolism and serve as an excellent tool for biomarker discovery. For example, Jain *et al.* analyzed the metabolic profiles of the NCI-60 panel of cancer cell lines and, using a data-driven approach, identified glycine consumption as a key metabolic driver of rapid proliferation in cancer cells (48). They found MTHFD2 expression to be among the topmost correlated with proliferation rates, thus demonstrating the power of metabolomic analyses to identify novel therapeutic targets.

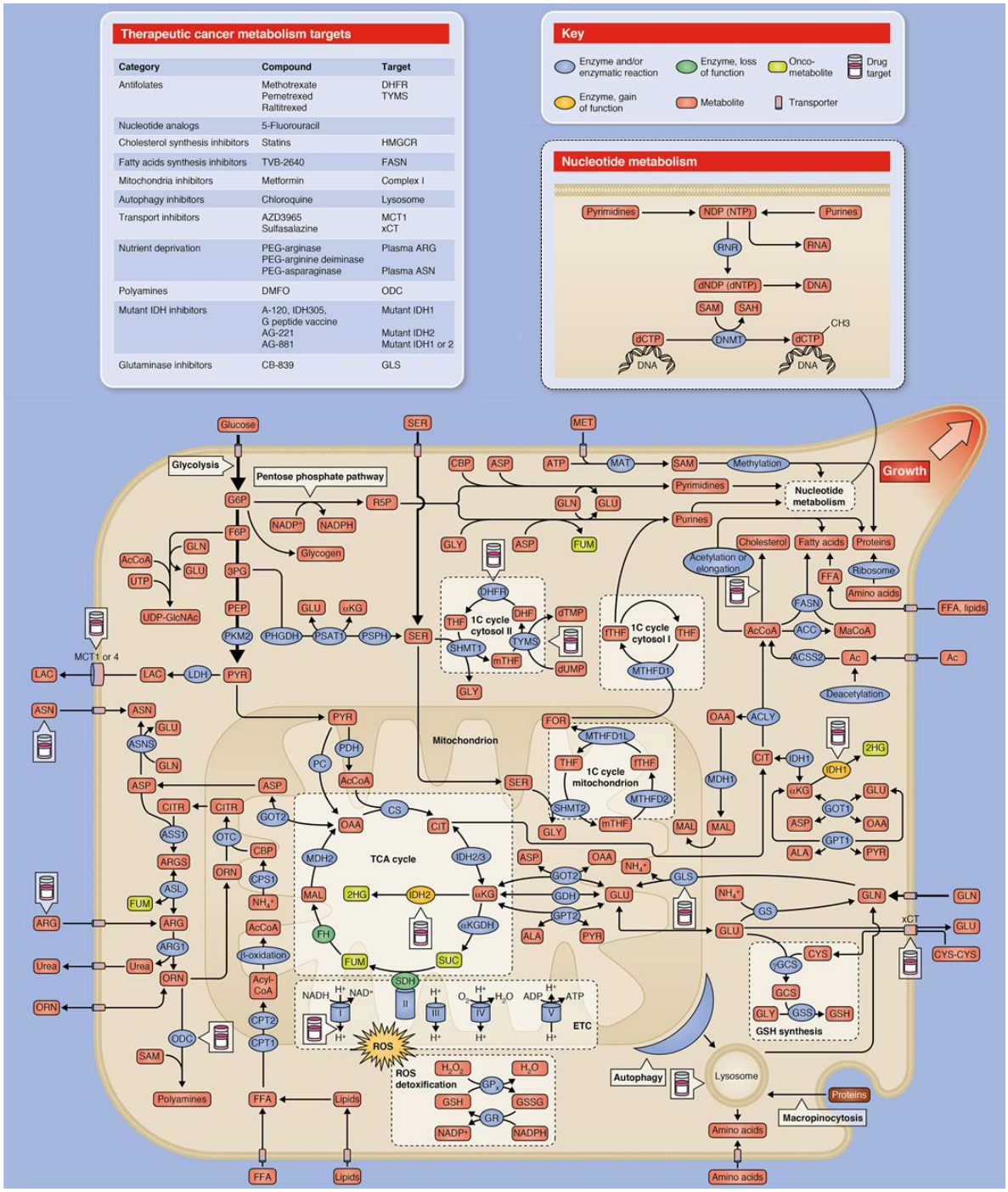

Figure 2. Overview of current therapeutic targets exploiting cancer metabolism. Cancer metabolism has been the target of cancer therapy since the early days of chemotherapy, with antifolates among the first targeted treatments. Our understanding of cancer metabolism has advanced significantly in recent years and is being used for the development of novel targeted therapies. Reprinted from Vazquez *et al.* 2016 (49). Copyright © 2016. Published by The Company of Biologists Ltd.

1.1.3 Glycolysis and PFKFB3

As the most abundant and ubiquitous source of energy, glucose and its metabolism are key determinants in the growth and expansion of dividing cells, and in particular tumor cells. Most of the glucose consumed by cells is normally catabolized through glycolysis to pyruvate, which fuels the TCA cycle and the electron transport chain (ETC) in aerobic cells (**Figure 3**). Glucose catabolism coupled to oxidative phosphorylation has a high energy yield in the form of ATP. Cancer cells, paradoxically, convert much of the pyruvate into lactate, which is then excreted to the extracellular medium, a phenotype known as the Warburg effect (25). Several glycolytic genes are usually overexpressed in tumors and give place to this effect (50). Even though catabolism of glucose into lactate has an extremely low energy yield, the percentage of ATP generated from glycolysis can surpass that produced by oxidative phosphorylation if the glycolytic flux is high enough (51–53). Furthermore, glucose metabolism provides intermediates that are needed for biosynthetic pathways, such as ribose sugars for nucleotide synthesis, acetyl-CoA for lipid production, non-essential amino acids and NADPH. Therefore, and as long as the rate is high enough, the glycolytic phenotype can fuel many of the cancer cell needs, e.g., energy, biosynthesis and redox balance (8).

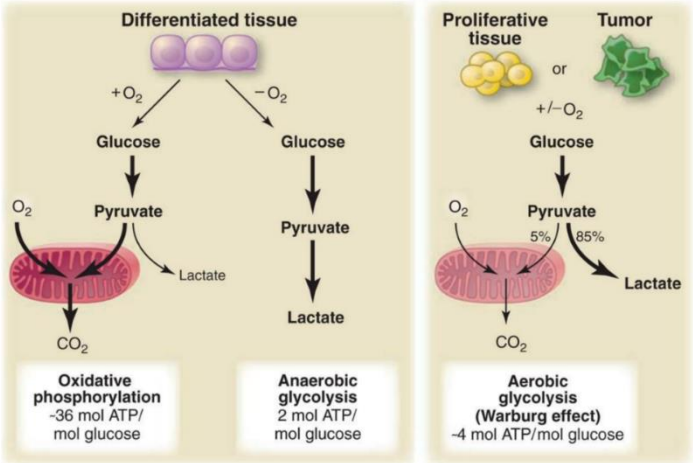

Figure 3. Differences in ATP output between oxidative phosphorylation, anaerobic and aerobic glycolysis. In aerobic conditions, differentiated tissues metabolize glucose to pyruvate via glycolysis and then shuttle most of it to the mitochondria for oxidative phosphorylation. Since oxygen is required as the final electron acceptor in the ETC, oxygen is indispensable for this process. In anaerobic conditions, cells can redirect the pyruvate generated by glycolysis into lactate production (anaerobic glycolysis). This allows glycolysis to continue (by cycling NADH back to NAD+) but results in minimal ATP production. Cancer cells tend to convert most glucose to lactate independent of oxygen availability (aerobic glycolysis). Mitochondria remain functional and oxidative phosphorylation can continue to varying extents in both cancer and normal proliferating cells. However, aerobic glycolysis remains significantly less efficient at generating ATP than oxidative phosphorylation. Reprinted from Vander Heiden *et al.*, 2009 (25), with permission from AAAS.

Glycolysis is tightly regulated by various mechanisms acting at different levels of the pathway (**Figure 4**). The first committed rate-limiting step of glycolysis involves the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP) by 6-phosphofructo-1-kinase (PFK-1), and represents a key regulation point in the pathway (54). Fructose 2,6-bisphosphate (F2,6BP) is a potent activator of PFK-1 (55), whose intracellular concentration is regulated by the family of bifunctional enzymes PFK-2/FBPase (PFKFB) (56,57). The four isozymes (PFKFB1-4), while sharing high sequence homology in their core domains, display quite different profiles when it comes to kinase-to-phosphatase activity ratios, tissue expression, and response to various signaling cascades (58,59). Among these isozymes, PFKFB3 has by far the highest kinase-to-phosphatase ratio (~740:1), which strongly promotes high glycolytic rates (60). Consequently, PFKFB3 has been shown to contribute to the Warburg effect and the hypoxia response (61–67), and therefore represents a promising target for anticancer therapy development.

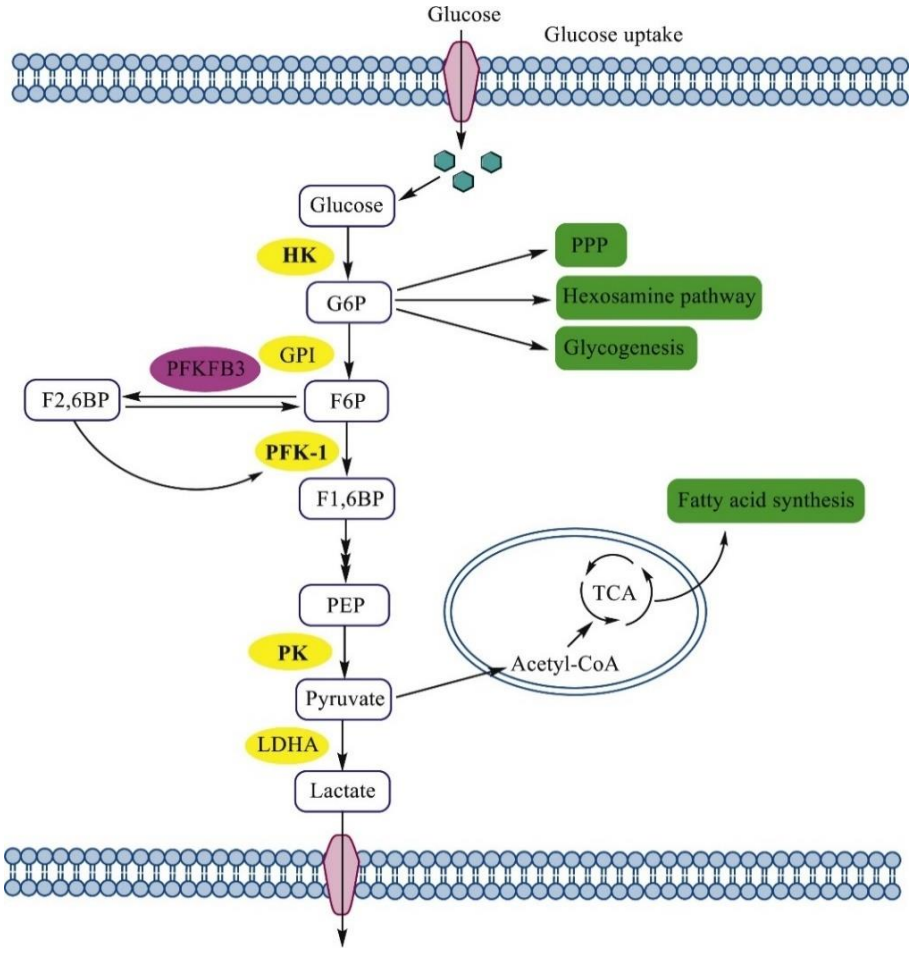

Figure 4. Overview of the main glycolysis steps in cancer cells. Catalytic enzymes for each step are shown in yellow or purple ovals. The three main modulating enzymes of the pathway are shown in bold. HK, hexokinase; G6P, glucose-6-phosphate; PPP, pentose phosphate pathway; GPI, glucose-6-phosphate isomerase; F6P, fructose-6-phosphate; F2,6BP, fructose-2,6-bisphosphate; PFK-1, phosphofructokinase 1; F1,6BP, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDHA, lactate dehydrogenase; TCA, tricarboxylic acid. Reprinted from Wang *et al*., 2020 (68), with permission from Elsevier.

PFKFB3 consists of two homodimeric subunits, each consisting of two functional domains (56,58,59). The C-terminal domain contains the bisphosphatase activity of the enzyme (69–71), while the N-terminal domain is responsible for its kinase activity (70). The relative lack of PFKFB3 phosphatase activity can be explained by a conformational rearrangement of C-terminal residues 440-446 which has not been observed in the other PFKFB isoforms (72). Several small molecule inhibitors of PFKFB3 have been developed, including 3PO, PFK15, PFK-158, with the latter having shown promising anticancer and immunomodulatory effects in clinical trials (73–76). However, the complex inhibitory mechanisms of these compounds and how they relate to their effect on different PFKFB3-mediated and -independent processes are still under investigation.

PFKFB3 protein expression is found in all tissues, but is specifically expressed at higher levels in adipose and brain tissue, as well as in transformed cells (77,78). PFKFB3 is frequently overexpressed in colon cancer, breast cancer, hepatocellular carcinoma, and glioma (79–82). Its expression is upregulated during the S phase of the cell cycle, and in response to hypoxia, inflammation and hormone receptor signaling (64,77,83,84). In addition to transcriptional control of PFKFB3 expression, various post-translational modifications of PFKFB3, e.g., phosphorylation, ROS-dependent S-glutathionylation, polyubiquitination, methylation and acetylation, can regulate its activity, stability and subcellular localization, allowing cancer cells to make rapid adaptive changes in response to metabolic stress (80,85–90).

In particular, PFKFB3 activity has been described as a direct link between oncogenic signaling and regulation of glucose metabolism in various tumor types (91,92). Upon mitotic arrest, prolonged energy deprivation activates AMP-activated protein kinase (AMPK), which phosphorylates PFKFB3 to promote glycolysis and evade cell death (93). In glioblastoma, inhibition of RAS signaling downregulates HIF-1α and thereby the expression of PFKFB3, hampering glycolysis and causing cell death (94). In breast cancer, constitutive HER2 signaling promotes PFKFB3 expression and increases glycolysis (95). In myeloid leukemias, PFKFB3 is transcriptionally repressed by myeloid translocation gene 16 (MTG16), thereby promoting mitochondrial respiration and inhibiting cell proliferation (96), while expression of PFKFB3 is activated by transcription factor PU.1 in chronic myeloid leukemia cells resistant to tyrosine kinase inhibitors (97). Moreover, loss of tumor suppressors such as p53 or PTEN has also been shown to result in activation of PKFBF3 and increased glycolysis (98,99).

Table 1. Overexpression of PFKFB3 in various cancer types

Cancer type	*Study material*	*Context*	*Reference*
Breast cancer	HMEC, MCF-10A, SKBR3, BT-474	*In vitro*	(95)
	HER2+ patient material	*In vitro*	(83)
	MCF-7, T47D	*In vitro*	(84)
	MCF-7, T47D, SUM159	*In vitro*	(100)
	Breast cancer patient material, MDA-MB-231, MDA-MB-438, HUVEC	*In vitro*	(101)
Melanoma	451LU, WM983	*In vitro*	(102)
	A375	*In vitro, in vivo*	(103)
	DB-1, SK-MEL-5	*In vitro*	(104)
Gastric cancer	MKN45, AGS, BCG823, GES-1	*In vitro, in vivo*	(74)
	MKN45, NUGC3	*In vitro*	(105)
Colon adenocarcinoma	Colorectal cancer patient material, SW480, SW1116	*In vitro, in vivo*	(106)
	HCT-116	*In vitro*	(107)
	FFPE tissue material, SW620	*In vitro*	(77)
	Panc1	*In vitro*	(105)
	Panc1	*In vitro*	(108)
Ovarian cancer	HeyA8, HeyA8MDR, OVCAR5, OV90	*In vitro*	(109)
Lung cancer	LLC1, H522	*In vitro*	(110)
	H522, H1437, PC9, HCC827	*In vitro*	(111)
Bladder cancer	T24, HUVEC	*In vitro*	(112)
Glioblastoma and astrocytoma	U87	*In vitro*	(104)
	Glioblastoma patient material	*In vitro*	(113)
	Glioblastoma and astrocytoma patient material	*In vitro*	(82)
	Glioblastoma and astrocytoma patient material, U87	*In vitro*	(114)
Head and neck carcinoma	HNSCC patient material, Cal27, FaDu	*In vitro*	(115)
Cervical cancer	OV2008, C13	*In vitro*	(109)
Renal cancer	ACHN	*In vitro*	(116)
Thyroid cancer	FFPE tissue material	*In vitro*	(77)
Osteosarcoma	U2OS	*In vitro*	(117)
	Osteosarcoma patient material, Saos-2	*In vitro*	(118)
Acute myeloid leukemia	THP-1, OCI-AML3	*In vitro*	(119)
Esophageal carcinoma	KYSE30, KYSE150	*In vitro*	(120)

In addition to its important role in glucose metabolism, PFKFB3 also carries out nuclear functions which support cancer proliferation independent from glycolysis (90). PFKFB3 possesses a highly conserved nuclear localization motif allowing its relocation to the nucleus, where it has been shown to indirectly promote G1/S cell cycle progression and prevent apoptosis through its activity on CDK4 stabilization and CDK1-mediated degradation of p27 (90,121–123). Furthermore, PFKFB3 has also been implicated in stimulating nucleotide synthesis for DNA repair in response to AKT and p53 signaling (81,124), as well as in HR repair of platinum-induced DNA damage (125), highlighting the existence of additional non-glycolytic roles for PFKFB3 in cancer cells.

To summarize, PFKFB3 plays a key role in the regulation of glycolysis, an important source of energy and biosynthetic intermediates on which cancer cells are particularly dependent on. The exploitability of this target for cancer therapy has been demonstrated by the development of potent PFKFB3 inhibitors with promising antitumor effects in clinical trials. Beyond its canonical role in glycolysis, nuclear functions of PFKFB3 may also be involved in promoting cancer development and require further investigation, to elucidate novel biological mechanisms and guide the development of additional therapeutic strategies using PFKFB3 inhibitors.

1.1.4 One-carbon folate metabolism and MTHFD2

Another consistently disrupted metabolic pathway in cancer is the folate cycle, which together with the methionine cycle constitute the one-carbon metabolic pathway (**Figure 5**) (48,126–128). Altered folate metabolism in cancer cells was among the first metabolic pathways to be targeted for cancer therapy, with pioneering studies by Sidney Farber in the late 1940s demonstrating that the folate analog aminopterin was capable of inducing remission in children with acute lymphoblastic leukemia (ALL) (129,130). These observations gave rise to new folate analogs, or antifolates, such as methotrexate (a WHO-designated essential medicine (131)) and pemetrexed, drugs which inhibit one-carbon metabolism and are still widely used today in cancer treatment (132–136). The early success of antifolates resulted in the advance of other types of antimetabolites, molecules which mimic natural substrates and inhibit their target enzymes, often involved in nucleotide synthesis (137–139). Since the 1950s, the widespread adoption and continuous use of antimetabolites such as 6-mercaptopurine, 6-thioguanine, 5-fluorouracil (5-FU), methotrexate, gemcitabine and cytarabine as standard-of-care agents for different cancer types has dispelled any doubts that folate and nucleotide metabolism are particularly attractive anticancer targets (140–143).

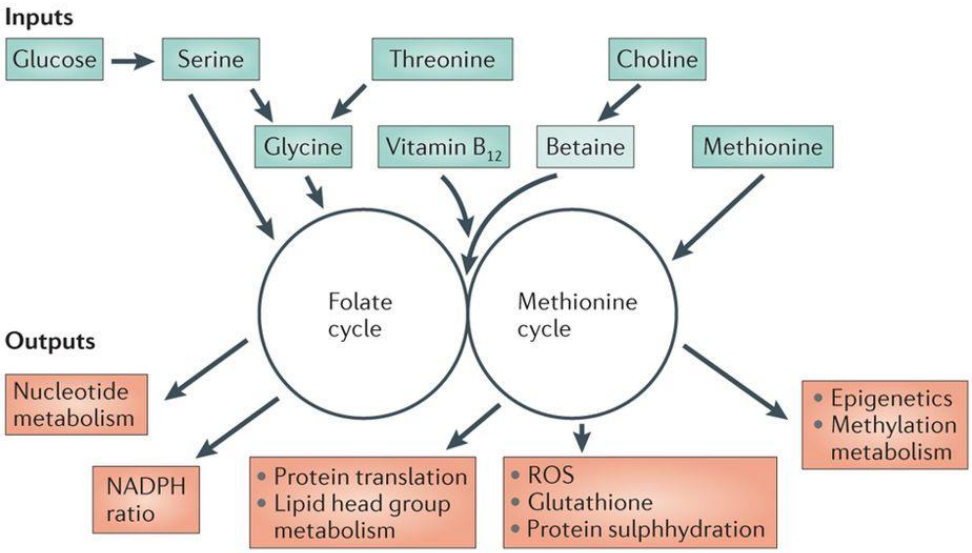

Figure 5. One-carbon metabolism is an integrator of nutrient status. One-carbon metabolism senses nutrient availabilities as cues to relocate resources into different biosynthetic pathways. It is composed of two modular units, the folate cycle and the methionine cycle. Depending on nutrient supply and demand, various outputs can be produced, including nucleotides, proteins, lipids, redox cofactors and substrates for methylation reactions. Reprinted by permission from Springer Nature: Nature Reviews Cancer, Locasale 2013 (128). Copyright © 2013, Nature Publishing Group, a division of Macmillan Publishers Limited. All rights reserved.

Indeed, rapidly growing and dividing cancer cells require more than just ATP to sustain their survival – large amounts of nucleic acids, proteins, lipids and redox cofactors also need to be produced to build new cells (7,24,25,52,144–146). One-carbon metabolism comprises a complex network of metabolic reactions which rely heavily on folate compounds and redox cofactors to activate, carry, and process carbon units to make them available for the production of nucleotides, amino acids, and lipids (147–150). The folate cycle is coupled to the methionine cycle through the generation of 5-methyl-THF (5-meTHF), which is used for the remethylation of homocysteine to generate methionine (151). Methionine can in turn be used for protein synthesis, as well as production of S-adenosylmethionine (SAM), the main methyl carrier substrate required for post-translational modifications and epigenomic maintenance (152–155). SAM is also required for the synthesis of phosphatidylcholine, creatine and polyamines (156–158). Together, the folate and methionine cycles are responsible for shunting carbon units into different biosynthetic pathways depending on specific cellular metabolic demands.

The folate cycle is fueled by dietary folic acid, which is reduced by dihydrofolate reductase (DHFR) to the biologically active tetrahydrofolate (THF) and disseminated in the circulation as THF-monoglutamate (159). Cells uptake monoglutamylated folates via active transport using the reduced folate transporter (RFC) (160). Upon entering the cells, folates are further polyglutamylated by folate polyglutamate synthetase (FPGS), which increases their affinity to folate enzymes and reduces their affinity for RFC, thereby ensuring intracellular retention (161,162). In the cells, THF acts as an acceptor scaffold for one-carbon units, becoming 5,10-methylene-THF (5,10-meTHF). Once loaded with carbon moieties, folates are unable to transfer across intracellular membranes – therefore, 5,10-meTHF must be generated in both the

cytosol and mitochondria independently (163). Serine and glycine are the main sources of one-carbon units, with many other molecules being able to produce glycine upon cleavage and demethylation (150,164,165). The transfer of one-carbon units from serine to THF is mediated by serine hydroxymethyltransferases (SHMT1 and SHMT2, cytosolic and mitochondrial isozymes respectively), while glycine can be used by aminomethyltransferase (AMT) to produce 5,10-meTHF. Glucose-derived carbon units from glycolysis can also be redirected into the folate cycle via de novo serine production through the action of pyruvate kinase M2 (PKM2) and phosphoglycerate dehydrogenase (PHGDH), among others (144,166–168). It has been known for many years that this pathway correlates with tumorigenesis, with PHGDH being commonly overexpressed in cancer (126,169–172).

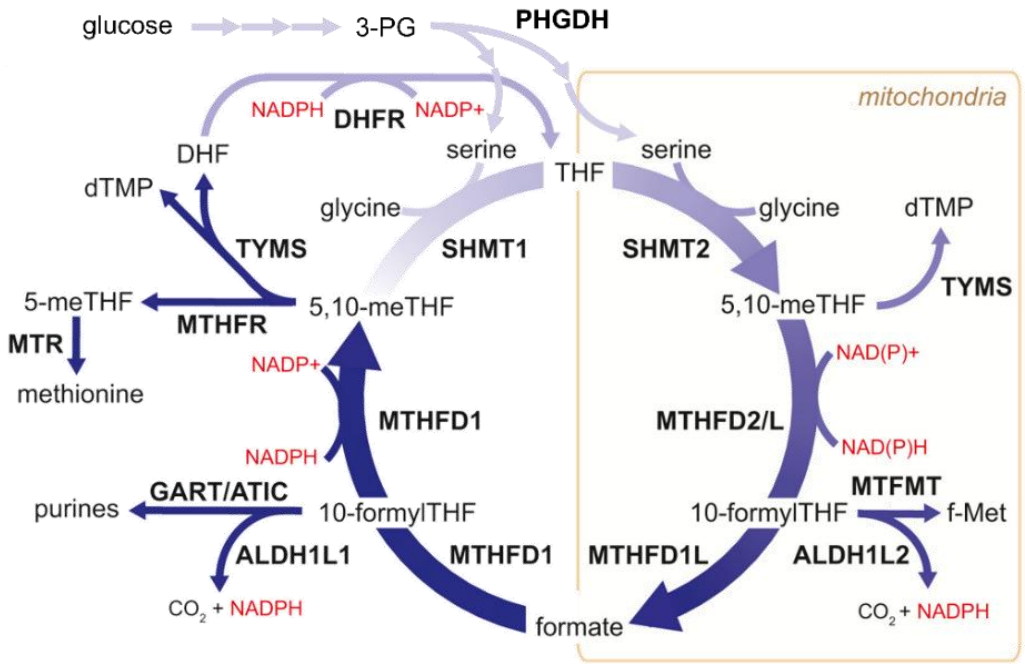

Figure 6. Folate-mediated one-carbon metabolism. In mammals, folate-mediated one-carbon metabolism is a highly compartmentalized and tightly regulated process. It possesses two main parallel branches, one mitochondrial and one cytosolic, each with its own set of specific isozymes and redox partners to convert between folate intermediaries. Interplay between the two modules is limited across the mitochondrial membrane. Adapted and reprinted from Ducker & Rabinowitz, 2016 (173), with permission from Elsevier.

The oxidation of 5,10-meTHF is a complex, multi-step process requiring three subsequent enzymatic activities: 5,10-methyl-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase, and 10-formyl-THF synthetase activities (**Figure 6**). This process is under additional spatiotemporal regulation, involving several different developmental and organelle-specific isozymes, each with different redox cofactor specificities. In the cytosol, interconversion between 5,10-meTHF and formate is carried out in its entirety by the trifunctional, NADP-dependent MTHFD1. In the mitochondria, however, the bifunctional (dehydrogenase, cyclohydrolase) NAD-dependent MTHFD2L together with the monofunctional (synthetase) MTHFD1L are responsible for catalyzing these reactions (174). In undifferentiated tissue or

during embryogenesis, the mitochondrial dehydrogenase and cyclohydrolase activities are carried out by MTHFD2 instead, as MTHFD2L is largely absent at early embryonic stages (175–178). Indeed, MTHFD2L seems to not have many crucial functions in highly proliferative tissues or cancer cells (179).

Supply and demand of specific metabolic units in proliferating cells can determine the direction of flow and fate of one-carbon intermediaries, as most folate enzymes catalyze bidirectional reactions (180,181). For example, 5,10-meTHF can be used to support DNA synthesis by the action of thymidylate synthase (TS; TYMS), which converts deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) (182). This is the target of standard-of-care anticancer agents such as 5-FU and raltitrexed (183–186). Alternatively, 5,10-meTHF can also be used for serine production by SHMT enzymes to satisfy high protein demands, or it can be fully oxidized to 10-formyl-THF for *de novo* purine synthesis (181,182,187,188). Moreover, folate cycle reactions can also produce redox cofactors, notably NADPH, which is important for maintenance of mitochondrial redox balance and has been implicated in cancer aggressiveness (189,190). Generally, mutations affecting genes involved in one-carbon metabolism often result in developmental defects in animals and humans, with most deletions being embryonic lethal, highlighting the crucial importance of this pathway for cell growth and proliferation (191–198).

As one-carbon metabolism constitutes a key source of building blocks required to sustain proliferation and maintain redox balance, its upregulation in cancer cells is unsurprisingly common. In the last few years, several genomics and metabolomics studies have singled two main one-carbon metabolism nodes, *de novo* serine and mitochondrial folate pathways, among the most upregulated metabolic pathways in cancer (48,126–128,199). The increased expression of one-carbon enzymes seems to stem mainly from altered transcriptional regulation rather than genomic amplification. ATF4 and mTOR have recently been described to modulate transcriptional regulation of both *de novo* serine synthesis and mitochondrial one-carbon enzymes (200,201). MYC and RAS have also been shown to induce transcription of genes involved in mitochondrial function (127,202–205). In KRAS-mutated non-small cell lung cancer, expression of MTHFD2 was shown to correlate with response to pemetrexed (206).

Among the metabolic genes commonly overexpressed in human tumors, MTHFD2 was identified as the most consistently upregulated metabolic enzyme across cancer types, as well as displaying the most cancer-specific expression profile (207). MTHFD2 overexpression levels have been associated with larger tumor size, increased metastasis and overall poor prognosis in breast cancer patients (208,209). Similarly, in acute myeloid leukemia (AML) *in vivo* models, loss of MTHFD2 alone was enough to impair cancer development and extend mouse survival (210). Furthermore, MTHFD2 has been shown to have nuclear localization and co-localize to DNA synthesis sites in cancer cells (211,212), not unlike other folate metabolism enzymes which rnrsupport dTMP production in the nucleus such as SHMT1, SHMT2α, MTHFD1 and TS (213–217).

Table 2. Antiproliferative effects of targeting MTHFD2 in different tumor types

Cancer type	*Study material*	*Method*	*Context*	*Reference*
Breast cancer	MDA-MB-231	siRNA	*In vitro*	(208)
	Hs578T, MCF-7	shRNA	*In vitro*	(207)
	MCF-7	shRNA	*In vitro*	(218)
	MCF-7	shRNA	*In vitro*	(219)
	MDA-MB-231	Small molecule	*In vivo*	(220)
Colon cancer	HCT-116, SW620, HCT15, HT29	shRNA	*In vitro*	(207)
	HCT-116	CRISPR/Cas9	*In vivo*	(181)
	HCT-116, Caco-2	siRNA	*In vitro*	(221)
	Colorectal cancer patient material, SW620, LoVo	shRNA, small molecule	*In vivo*, PDX	(222)
	HCT-116, HT29	siRNA	*In vitro, in vivo*	(223)
Lung cancer	HOP92, H226, EKVX, H460	shRNA	*In vitro*	(207)
	Adenocarcinoma patient material, H322	shRNA	*In vitro, in vivo*	(224)
	A549, H1299	shRNA	*In vitro, in vivo*	(225)
	A549, H1299, H441	shRNA	*In vitro, in vivo*	(226)
Acute myeloid leukemia	THP-1, MV4-11, MOLM-14, U937	shRNA	*In vitro, in vivo*	(210)
	HL-60, THP-1	siRNA	*In vitro*	(227)
	HL-60, THP-1	siRNA	*In vitro*	(228)
Renal cancer	A498	shRNA	*In vitro*	(207)
	786-O	shRNA	*In vitro*	(229)
	786-O, CAK-1	shRNA	*In vivo*	(230)
Glioblastoma	U251, SNB-75, SF295	shRNA	*In vitro*	(207)
	U87, U118	siRNA	*In vitro, in vivo*	(231)
Neuroblastoma	SK-N-DZ	shRNA	*In vitro*	(232)
Ovarian cancer	OVCAR8	shRNA	*In vitro*	(207)
Melanoma	LOX IMVI	shRNA	*In vitro*	(207)
Liver cancer	HepG2, Huh7	siRNA	*In vitro*	(233)

Altogether, MTHFD2 has been extensively validated as a promising anticancer target given its highly cancer-selective profile, the widespread expression of redundant MTHFD2L in normal tissue, and its function in supporting nucleotide synthesis in cancer. Toxicity and side effects of current antimetabolites could potentially be curbed by favoring MTHFD2 inhibition instead, thereby significantly improving the therapeutic window of antifolate strategies.

1.2 DEREGULATION OF NUCLEOTIDE POOLS AND GENOME INSTABILITY

1.2.1 Nucleotide synthesis pathways

The maintenance of genomic integrity is paramount for cell survival. Thus, considerable cellular resources and mechanisms are dedicated to preventing damage to the DNA from endogenous and exogenous insults, such as ROS, replication stress, carcinogens, radiation, etc. Genomic alterations are common drivers of tumor initiation and progression, and as such, cancer cells are characterized by higher baseline levels of genomic instability (22,234–236). Increased genetic instability enables higher mutational rates to promote tumor development, heterogeneity and plasticity, however it also renders cancer cells more dependent on certain genomic maintenance mechanisms, thereby sensitizing them to targeted therapies against DNA replication, repair and checkpoint pathways (237–241).

A key aspect of genome maintenance is to ensure adequate levels of deoxynucleotide reserves and correct incorporation during DNA replication or repair (242,243). Deregulated nucleotide metabolism has long been associated with oncogenesis, with studies showing that decreased nucleotide pools are sufficient to induce genomic instability and mutagenesis (244–248). Nucleotides can be synthesized *de novo*, regenerated through salvage pathways or scavenged from the environment. Given the increased and sustained requirement for nucleic acids in tumor cells, salvage pathways alone are unable to satisfy this demand (249). Key enzymes involved in nucleotide metabolism, such as TS, RNR, inosine-5'-monophosphate dehydrogenase (IMPDH), glycinamide ribonucleotide formyltransferase (GARFT), and dihydroorotate dehydrogenase (DHODH), are commonly upregulated in cancer and constitute primary targets for anticancer therapy (**Figure 7**) (250,251,260,252–259).

Nucleotide metabolism is highly dependent on upstream mTOR and MYC signaling (261,262), which control the expression and post-translational modifications of process-initiating enzymes such as phosphoribosyl pyrophosphate synthetase 2 (PRPS2), carbamoyl-phosphate synthetase 2-aspartate transcarbamoylase-dihydroorotase (CAD), and auxiliary enzymes such as PFKFB3 and MTHFD2, thereby modulating the substrate inputs stemming from the pentose phosphate pathway or the folate cycle (119,124,200,263–265).

Of particular importance is the regulation of dTMP metabolism enzymes, to ensure sufficient supplies of dTTP and prevent incorporation of uracil into the DNA during replication, or more generally to avoid thymineless death (245,266). In eukaryotic cells, DNA polymerases are unable to distinguish between dUTP and dTTP, therefore dUTP/dTTP ratios are tightly controlled and kept low, between 0.1% to 3%, to prevent uracil misincorporation (267). Misincorporated uracil can also be removed by uracil-N-glycosylase (UNG) and base excision repair (268), yet excessive DNA excision can lead to replication stress and genomic instability. Mutagenesis caused by RNR overexpression has been shown to stem from an increased incidence of erroneous nucleotide incorporation by DNA polymerases (269). High levels of dUTPase in cancer patients correlate with poor response to TS inhibitors 5-FU and

fluorodeoxyuridine (5-FUdR), and have been shown to become upregulated during the development of resistance (270–272). Nuclear localization has been described for both RNR and dUTPase, as well as TS, SHMT1, SHMT2α, MTHFD1 and MTHFD2, highlighting the importance of local thymidylate synthesis and dUTP exclusion close to DNA replication and repair sites (211,215,216,273–275).

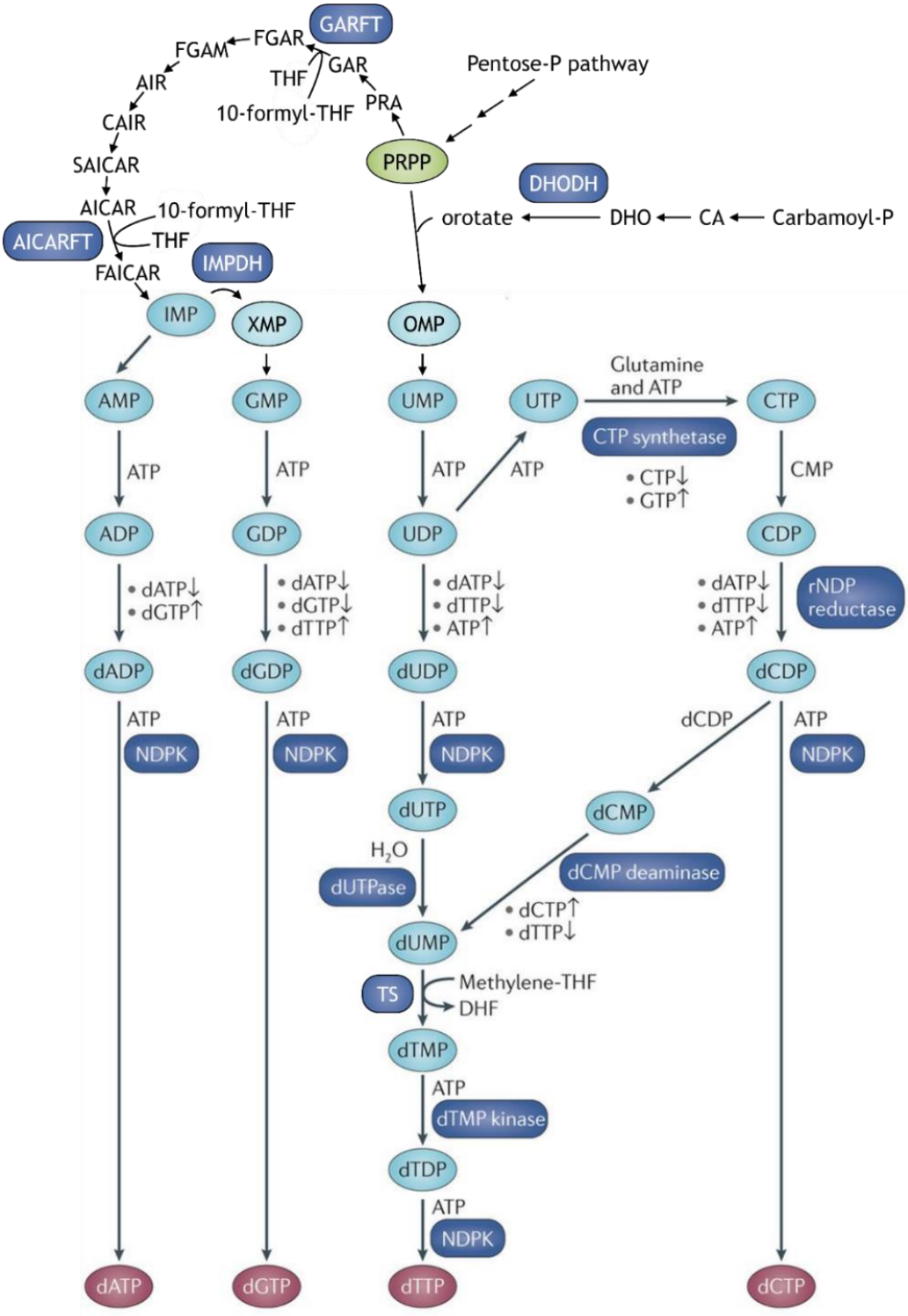

Figure 7. *De novo* purine and pyrimidine synthesis for production of dNTPs. Ribose-5-phosphate from the pentose phosphate pathway is transformed into its active form, 5-phosphoribosyl-1-pyrophosphate (PRPP), required for both purine and pyrimidine synthesis. Purine synthesis is spatially organized in multienzyme complexes called purinosomes and follows a series of ten amidotransferase and transformylation reactions, consuming ATP, glutamine, glycine, aspartate, CO_2 and 10-formyl-THF, culminating in the formation of inosine 5'-monophosphate (IMP), the precursor to AMP and GMP. Pyrimidine synthesis is less complex as the base is much simpler than purines. First, glutamine is used as a nitrogen donor as carbamoyl phosphate, followed by incorporation of aspartate to form carbamoyl aspartate. The enzymes dihydroorotase and DHODH convert it into orotate, which is finally conjugated to the ribose from PRPP to form orotate monophosphate (OMP) and UMP. UMP can then be used to form dCTP and dTTP (276). The rate-limiting step of dNTP synthesis is catalyzed by RNR to form dNDPs, followed by nucleoside diphosphate kinase (NDPK) phosphorylation yielding dNTPs (277). Synthesis of dTTP requires additional steps catalyzed by dUTPase, cytidine deaminase (CDA), TS and dTMP kinase (TMPK), consuming an additional equivalent of 5,10-meTHF. Adapted and reprinted by permission from Springer Nature: Nature Reviews Cancer, Mathews 2015 (248).

1.2.2 Altered metabolism and replication stress

Due to its fundamental importance for genome stability, DNA replication is one of the most highly regulated processes in cells. Replication stress, i.e., any condition affecting the precise and timely progress of DNA replication, is a main source of genomic instability in cancer cells (278–283). Histone and dNTP shortages, hard to replicate sequences, and collisions between replication and transcriptional machineries can all cause replication stress (244,279). Stalled replication forks resulting from these conditions activate the replication stress response, or S phase checkpoint, which includes cell cycle arrest, reduced firing of new replication origins, activation of dNTP production and DNA repair pathways to enable the restart of stalled replication forks and continuation of cell cycle progression (280,283,284). The ataxia telangiectasia and Rad3-related (ATR) kinase is the main regulator of the replication stress response, and together with mTOR and checkpoint kinase 1 (CHK1), coordinates the timely execution of these processes (285–287).

mTOR plays an important role under replication stress conditions, where it is directly phosphorylated by ATR and upregulates expression of CHK1 to enhance the replication stress response. Activation of mTOR stimulates anabolic nucleotide synthesis through expression of CAD, MTHFD2 and RNR, but also balances catabolic processes including cell cycle arrest, cell death and autophagy (201,288). Beyond its canonical function as a main nutrient sensor and metabolic modulator, mTOR also directly regulates cell cycle through the transcriptional and translational regulation of cyclins and cyclin dependent kinases (CDKs) (289–294). Combination of mTOR inhibitors with traditional chemotherapies inducing replication stress such as topoisomerase inhibitors has demonstrated great anticancer activity *in vitro* and *in vivo*, with several combination regimens currently being tested in clinical trials (295–299). More recently, there has been tremendous efforts to selectively target high levels of replication stress in cancer cells using ATR-CHK1 pathway inhibitors (241,300,301), broadening our therapeutic opportunities via synthetically lethal DNA replication challenges to cancer cells.

1.2.3 Homology-directed repair of DSBs

DNA molecules are exposed to various intrinsic and external sources of damage on a permanent basis. Therefore, maintenance of genome integrity via prompt and accurate DNA damage repair is crucial for cells to ensure passing on intact genetic code to the next generation of cells and prevent the rise of genetic abnormalities (234,302). Upon DNA damage, cells activate different signaling cascades eliciting specific repair machineries depending on the type of damage incurred.

DSBs are particularly toxic DNA lesions, which can lead to replication stress and cell death if left unrepaired (303–305). When DSBs arise, cells can choose between two main repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair, also called HR repair (**Figure 8**) (306–308). The choice of pathway is largely controlled by cell cycle phase-dependent DNA end-resection mechanisms which commit the repair to the error-free HR pathway when a sister chromatid template is present in S and G2/M phases, or relegate it to error-prone NHEJ which directly ligates the DSB ends and generates potentially mutagenic insertions and deletions (309,310).

DDR signaling is orchestrated by a family of phosphoinositide 3-kinase (PI3K)-related kinases: DNA-PK, ATM and ATR (311). Repair of DSBs involves mainly ATM and DNA-PK signaling, with activation of ATM by the DNA damage sensing complex Mre11-Rad50-Nbs1 (MRN) and subsequent DNA end-resection initiating HR repair (308,310,311). Deregulations in DNA repair pathways, including mismatch and HR repair mechanisms, are common in cancer and contribute to their development (234,312). Efforts to exploit these DDR defects in cancer therapy have proven extremely successful under certain genetic and cellular contexts, however the rise of resistance severely hinders the clinical benefit of these therapies (241,313,314). New approaches to expand the scope and benefit of therapeutic strategies which target the DDR and HR deficiency hold a transformative potential for cancer patient care.

The strong link between DNA repair and cellular metabolism has become gradually more apparent in recent years, particularly in cancer cells, where metabolic deregulations have been shown to both promote mutagenic DNA damage as well as support DNA repair pathways (**Figure 9**) (315,316). Many new metabolic players have been identified as having promoting roles in DSB repair (11,317–319), either through direct interaction with core DNA repair proteins, modulation of nucleotide pools, or regulation of chromatin remodeling via epigenetic markers (316).

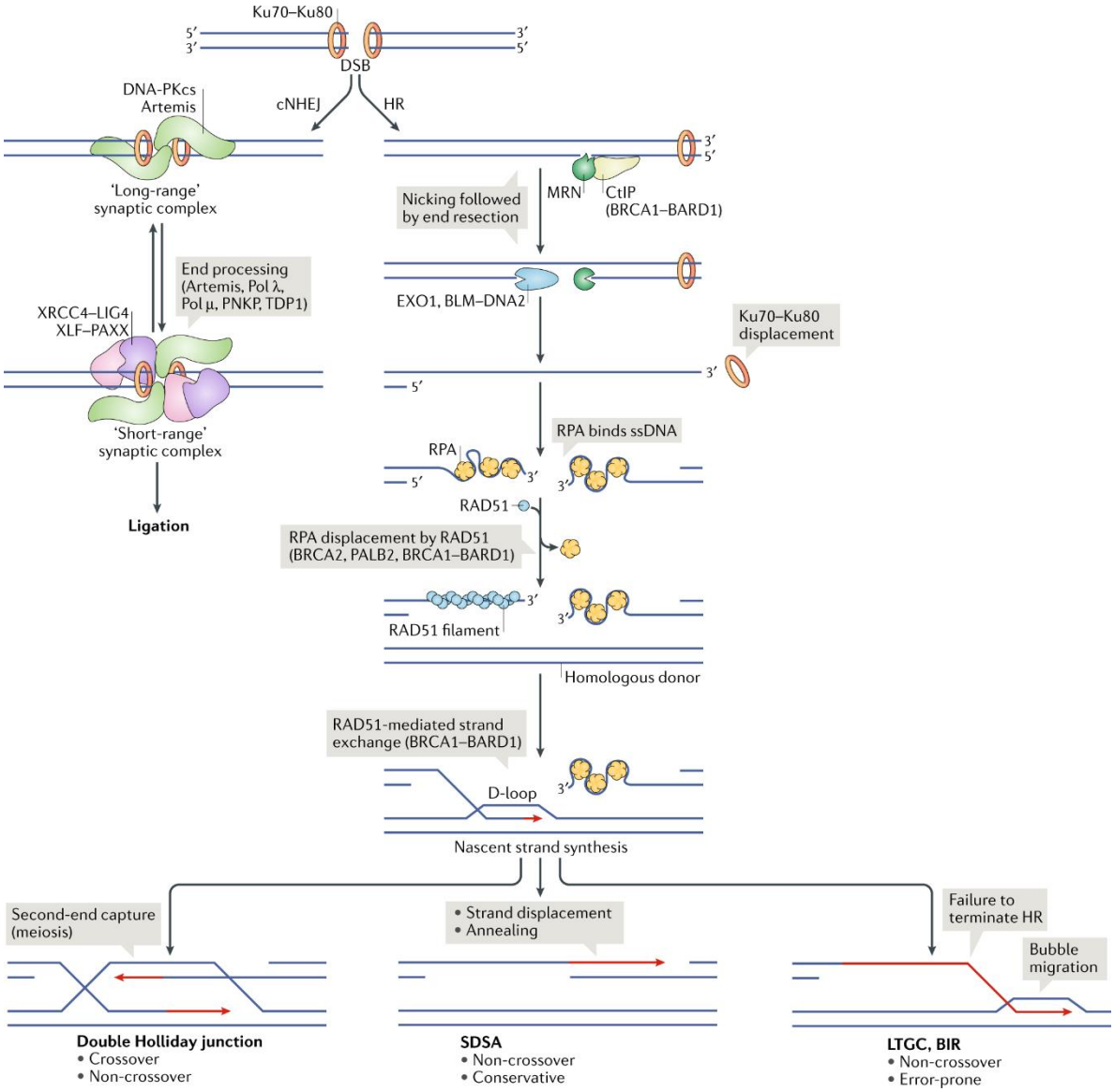

Figure 8. Choice of repair pathway for DSBs. While NHEJ proteins Ku70/Ku80 and 53BP1 bind DSBs across the cell cycle by default and prevent DNA resection, BRCA1 acts as a repressor of 53BP1 in S and G2/M phases to promote HR repair. The endonuclease activity of Mre11 then initiates resection together with CtIP, which is further extended by exonuclease 1 (EXO1) and the DNA2-Bloom syndrome protein (BLM) heterodimer, releasing the Ku complex from the DNA. The ssDNA overhangs are rapidly bound and stabilized by RPA, which is later displaced by RAD51 with the help of mediators BRCA2, PALB2 and the BRCA1–BRCA1-associated RING domain protein 1 (BARD1) complex. The RAD51 filaments are then able to guide homology query of the genome and strand invasion. Following homology pairing and nascent strand synthesis by DNA polymerases δ and ε, D-loop structures are primarily resolved by synthesis-dependent strand annealing (SDSA), which does not involve cross-over and thus prevents loss of heterozygosity. Other resolution modalities exist such as double Holliday junctions which promote crossovers during meiosis, as well as backup yet error-prone long-tract gene conversion (LTGC) and break-induced replication (BIR). Reprinted by permission from Springer Nature: Nature Reviews Molecular Cell Biology, Scully *et al.*, 2019 (308). Copyright © 2019, Springer Nature Limited.

Proper maintenance of nucleotide pools is particularly important for DNA replication, but is also necessary for repair DNA synthesis, as shown by the cellular need to produce dNTPs at DNA damage sites via the recruitment of RNR and thymidylate kinase (TMPK) for example (245,274,288). While the relative amount of nucleotides required for repair is small compared to DNA replication demands, local dNTP concentration is critical as cells cannot rely solely on nucleotide diffusion to ensure adequate repair. In line with this, *de novo* dNTP production has been identified as a key factor in the choice of DSB repair pathway, with HR relying more heavily on RNR activity and nucleotide synthesis than NHEJ, as it involves more extensive DNA synthesis (320). Moreover, upon DNA damage, ATM activates G6PD to promote metabolic rewiring towards the PPP to create more nucleotide precursors, while radioresistant cancer cells have been shown to upregulate GS to divert glucose and glutamine metabolism towards the production of dNTP (321,322). Finally, both phosphoglycerate mutase 1 (PGAM1) and PFKFB3 have also been found to regulate dNTP pools: PGAM1 through modulation of 3-PG/2-PG balance in favor of PPP, and PFKFB3 by recruiting RNR to DSB sites (319,323).

Other enzymes have been shown to have direct effects on DNA repair factors. In fact, some enzymes can have different effects on separate fronts, such as PGAM1, which in addition to supporting PPP, can indirectly modulate CtIP degradation via 3-PG inhibition of 6-phosphogluconate dehydrogenase (6PGD) (323). Another example is PKM2, which is retained in the nucleus following DSB-induced ATM phosphorylation, and contributes to the phosphorylation and activation of γH2AX and CtIP to amplify DDR signaling and HR repair (317,324). Besides glycolytic factors, ornithine decarboxylase (ODC) is the rate-limiting enzyme in polyamine production and is commonly upregulated in cancer, in part due to polyamines promoting HR via RAD51-mediated strand invasion and contributing to chemotherapy resistance (325,326).

Finally, chromatin accessibility and epigenetic remodeling are heavily dependent on metabolic input and can also influence the choice of repair pathway, either through direct modulation of chromatin remodelers or the availability of methyl- and acetyl-group donors necessary for these reactions. Acetyl-CoA producing enzyme ATP-citrate lyase (ACLY) is overexpressed in tumors, is activated by ATM and AKT following IR and localizes to the nucleus to supply acetyl-CoA at DSB sites and favor BRCA1 recruitment over 53BP1 by histone acetylation, thus promoting HR (327–330). Accumulation of oncometabolites 2HG, succinate and fumarate inhibit lysine demethylases (KDMs) resulting in histone hypermethylation which prevents recruitment of HR DNA repair factors (331–336). Increased glucose and glutamine metabolism result in higher N-acetyl-glucosamine (GlcNAc) production and protein O-GlcNAcetylation, which activates repair-promoting factors such as enhancer of zeste homolog 2 (EZH2), a histone-lysine N-methyltransferase responsible for pro-NHEJ H2K27 methylation (337).

From all this evidence, it is clear that metabolic rewiring in cancer cells protects them against radio- and chemotherapy by increasing their DNA repair potential, and thus presents a targetable vulnerability that can be exploited in combination strategies.

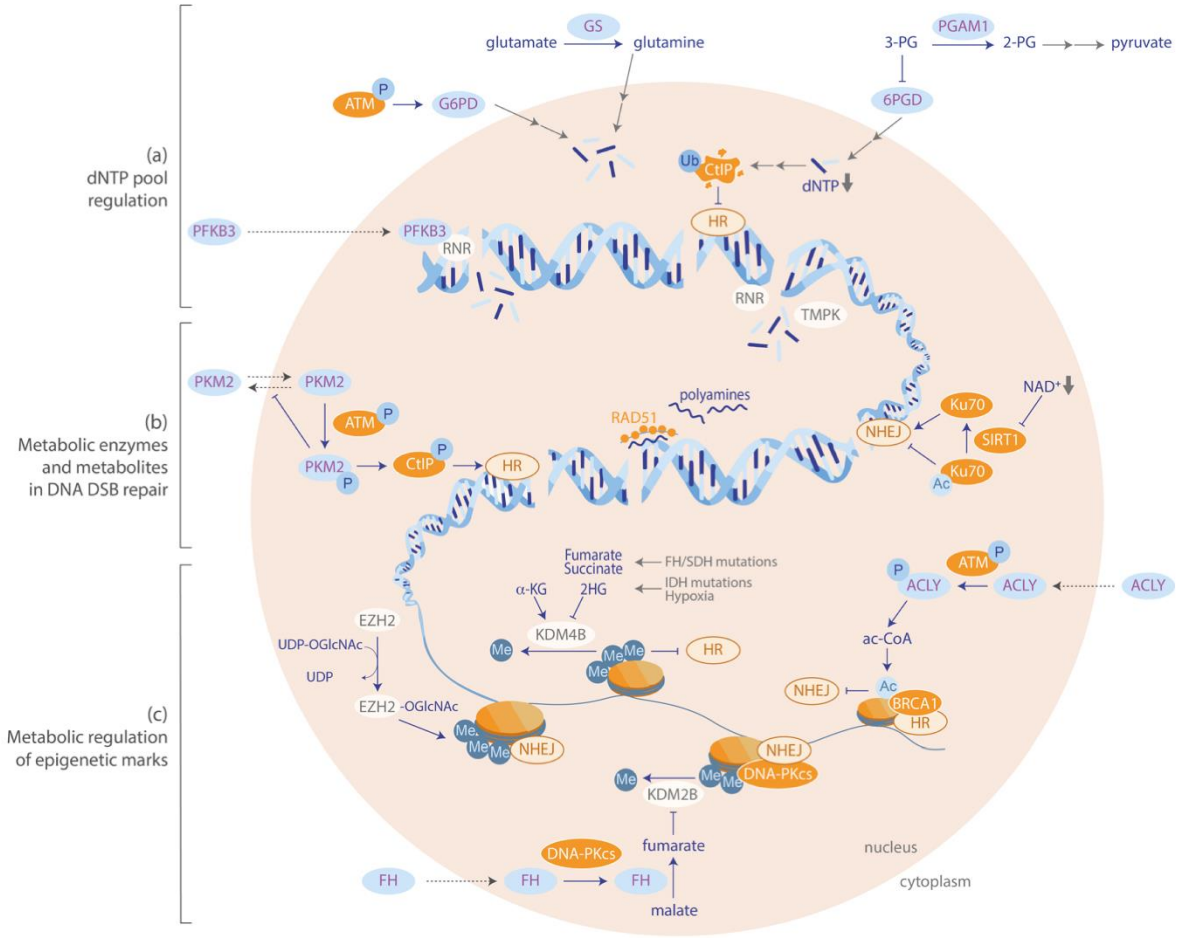

Figure 9. Metabolic modulation of DSB repair. The interplay between DNA repair and metabolism can be organized into three main types: (a) dNTP pool regulation, (b) direct involvement in DNA repair, and (c) regulation of epigenetic chromatin remodeling. 6PGD, 6-phosphogluconate dehydrogenase; ACLY, ATP-citrate lyase; ATM, ataxia-telangiectasia mutated kinase; BRCA1, breast cancer type 1; CtIP, CTBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; EZH2, enhancer of zeste homolog 2; G6PD, glucose-6-phosphate dehydrogenase; GS, glutamine synthetase; KDMB2/4B, lysine demethylases 2B and 4B; PGAM1, phosphoglycerate mutase 1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM2, pyruvate kinase M2; RNR, ribonucleotide reductase; SIRT1, NAD-dependent deacetylase sirtuin-1; TMPK, thymidylate kinase. Reprinted from Moretton & Loizou, 2020 (316). Copyright © 2020. Published by MDPI.

1.3 COMBINATION STRATEGIES TARGETING THE DDR IN CANCER TREATMENT

1.3.1 Radiosensitization

Radiation therapy, or radiotherapy, constitutes one of the most established and successful anticancer therapies still used to this day. Ionizing radiation (IR) creates a variety of DNA lesions which activate cell cycle arrest and, if left unrepaired, can cause cell death (338,339). As such, it is particularly detrimental for rapidly dividing cells such as tumor cells, but also normally renewing healthy tissue like intestinal epithelia and hematopoietic cells. This unintended toxicity is often dose limiting, with suboptimal radiation doses potentially incurring

in further mutagenesis, thus promoting the emergence of resistance mechanisms and tumor escape (340). The more radiosensitive tumors are relative to the surrounding normal cells, the better the therapeutic effect (341). Although much emphasis has been put on protecting the healthy tissue (341), an attractive and complementary alternative would be to disable DNA repair mechanisms specifically in cancer cells to render them incapable of coping with radiation-induced DNA damage.

Radiotherapy induces many different types of genomic lesions, by directly damaging the phosphodiester backbone of DNA, or indirectly via the creation of free radicals and ROS (339,342). While single strand breaks (SSBs) are more frequent, double strand breaks (DSBs) have been demonstrated to be the most lethal (303–305). As mentioned previously, the DNA damage response (DDR) to DSBs preferentially involves the error-free HR pathway when a sister chromatid is available to serve as template for repair in late S an G2/M phases. Therefore, cancers which are HR-deficient can be intrinsically more sensitive to radiotherapy, while HR-proficient tumor cells may become sensitized by concomitant targeting of HR-supporting pathways. Combining conventional chemotherapy, as well as targeted DDR inhibitors, with radiation therapy has proven successful in increasing its effectiveness (343,344). However, many hypoxic solid tumors present a particular challenge as their low-oxygen niche affects radiation efficacy by reducing the amount of oxygen free radicals produced (345), which is why multiple oxygen therapeutic compounds are currently under clinical investigation to overcome this issue (346–349).

Metabolic rewiring has been shown to mediate radiotherapy resistance by promoting a glycolytic phenotype, allowing cancer cells to thrive in hypoxic conditions (350,351). Thus, targeting glycolysis which is crucial for energy production in proliferating tumor cells via one of its main regulators in cancer, PFKFB3, represents a promising therapeutic avenue to expand the range and improve the selectivity of current treatment options, even for slow-growing cancers (78,352–355).

1.3.2 Pioneer and new DDR synthetic lethal strategies in the clinic

Cancer development is the result of a progressive multistage accumulation of genetic mutations, yet previous studies have shown that cancer survival can often be affected by targeting a single oncogene, a phenomenon known as oncogene addiction (356–359). Oncogene addiction has been the target of many anticancer strategies, with targeted therapies being adopted as standard-of-care anticancer treatment and proven successful even against tumors which fail to respond to standard chemotherapy. Nevertheless, precision genotype-targeted cancer treatments are often limited by the rapid development of resistance and patient relapse. This prompted the exploration of secondary targets which would become required for cancer cell viability only in the specific context of the tumor’s mutational landscape, i.e., synthetic lethality (360).

Since the discovery of genetic alterations as tumorigenic drivers, genes involved in DNA synthesis, maintenance and repair have been considered *bona fide* tumor suppressors, as loss of proper DNA metabolism often leads to oncogenic mutations and genomic rearrangements. This is well exemplified by familial breast and ovarian cancer driven by BRCA1 or BRCA2 mutations, both well-known factors of homologous recombination DNA repair pathways, or hereditary colorectal cancer caused by defects in mismatch repair proteins (361,362). Undoubtedly, the most successful example of synthetic lethality being exploited for anticancer therapy is the development of poly-ADP-ribose polymerase (PARP) inhibitors for the treatment of BRCA-deficient tumors (313,363,364). Numerous statistical prediction and genetic screen studies using RNAi and CRISPR/Cas9 have uncovered additional synthetic lethal combinations with great potential for clinical targeting, prominently featuring DNA damage sensing and repair pathways (365–368).

Today, a large fraction of anticancer drugs used in the clinic induce replication stress, including traditional genotoxic agents such as topoisomerase inhibitors and platinum compounds, as well as antimetabolites affecting nucleotide synthesis. Moreover, increasing the replication stress burden by the combined action of genotoxic drugs and nucleotide analogues has shown increased anticancer efficacy (369). However, the activation of cell cycle checkpoints in response to these treatments does not necessarily translate into cancer cell death. Due to their high intrinsic load of oncogene-induced replication stress, cancer cells are particularly reliant on proficient replication stress response mechanisms to survive under high replication stress conditions, achieved by overexpression of CHK1 and RNR for example (244,370,371). As replication stress is primarily detected by ATR, which also coordinates the mitotic entry checkpoint, loss of ATR in high replication stress conditions would remove the barriers preventing mitotic catastrophe and cell death (372–375). Indeed, tumors with high levels of replication stress have been shown to be particularly dependent on ATR signaling for survival, prompting the development and clinical evaluation of various ATR and CHK1 inhibitors (376–379). Based on this observation, levels of CHK1 in cancer cells have been used to predict response to ATR inhibition, identifying hematological cancers as particularly good candidates for ATR inhibition therapy, given their high expression of CHK1 (380–383). Further studies will be needed to evaluate the potentially beneficial combinations of ATR inhibitors beyond existing chemotherapies, particularly in the context of recent metabolic targets such as IDH, PFKFB3, PKM2, SHMT1 and MTHFD2 (34,167,384–386), in hematological malignancies as well as other tumor types.

1.3.3 Therapeutic trends in glycolysis and one-carbon metabolism

1.3.3.1 PFKFB3 and glycolytic inhibitors

The Warburg effect constitutes the most predominant metabolic hallmarks differentiating cancer cells from healthy tissue. Consequently, sizable efforts have been directed towards targeting increased glycolysis and lactate metabolism in cancer therapy (387–391).

One of the first and best characterized glycolytic inhibitors is 2-deoxyglucose (2-DG), which is phosphorylated by hexokinase (HK) into a non-metabolizable product that accumulates in the cells and inhibits HK to reduce glucose uptake (392). While preclinical evaluation of 2-DG showed promising antiproliferative effects, clinical use of this drug was limited by hypoglycemic toxicity, a result shared by many other glycolytic inhibitors (393–396). Clinical success of glucose uptake or lactate production targeting therapies remains limited despite encouraging results in preclinical models, highlighting the need for more precise targeting of glycolysis in cancer (15,27,389).

Since the first reports of tumor dependence on PFKFB3 upregulation started to suggest it as an attractive anticancer target, there has been a heightened interest in developing and evaluating PFKFB3 inhibitors to specifically target cancer cell glycolysis (385). The best-studied small molecule inhibitor described to target PFKFB3 is 3PO, which is a potent but poorly soluble inhibitor of PFKFB3, making it a difficult candidate for clinical development (73). A derivative of 3PO, PFK15, was later developed and shown to have improved pharmacokinetic properties and good selectivity over other glycolysis enzymes, demonstrating antineoplastic effects in gastric cancer, hepatocellular carcinoma and lung carcinoma *in vitro* and *in vivo* models (74,81,110). An improved analog of PFK15, PFK-158, has also shown great antitumor efficacy in various solid cancer models and displayed promising results in Phase I clinical trials against solid tumors (75,76,109,397). As a result of their structural differences, these compounds may have diverse pharmacokinetic and activity properties, and thus different effects on cancer cell metabolism. Importantly, new evidence has shown that 3PO and its derivatives PFK15 and PFK-158 do not bind PFKFB3, and that their anticancer effects may not be PFKFB3-mediated (398–400). Another promising class of PFKFB3 inhibitors developed by AstraZeneca displayed potent inhibition of PFKFB3 and reduction of F1,6BP production in A549 cells (398). While pharmacokinetic profiling of their lead compound showed acceptable properties (e.g., high oral bioavailability and moderate half-life), selectivity against the closely related PFKFB4 isoform was not reported and *in vivo* efficacy has yet to be determined. Numerous other efforts to develop PFKFB3 inhibitors have also contributed to our understanding of SAR and different inhibitory modalities, and highlight the need for compounds with improved isoform selectivity and *in vivo* stability (68). Studies describing PFKFB3 inhibitors in sensitizing or synergistic combinations with other chemotherapies indicate that these compounds may have great adjuvant applications, and endorse the continued development of structurally diverse PFKFB3 inhibitors to expand the repertoire and personalization potential of future therapeutic strategies (385).

1.3.3.2 MTHFD2 and serine/one-carbon metabolism inhibitors

Targeting one-carbon metabolism led to the first chemotherapeutic agents in the 1950's and 60's, some of which are still used today, such as methotrexate, pemetrexed and 5-FU. Overexpression of DHFR and TS, as well loss of FPGS and RFC function, are common mechanisms of acquired resistance to these antifolates. Moreover, their therapeutic efficacy is substantially limited due to toxicity caused by inhibition of one-carbon metabolism in non-transformed dividing cells, such as those in the intestinal lining and bone marrow, resulting in gastrointestinal side-effects, anemia, and compromised immunity. To curb these adverse effects, rescue therapy with 5-formyl-THF (known as folinic acid or leucovorin) is commonly given together with antifolate treatment, yet their therapeutic index remains moderate.

Given the increased dependence of tumor cells on this pathway, targeting *de novo* serine synthesis or mitochondrial folate metabolism would be a viable solution to selectively target one-carbon metabolism in cancer (401,402).

As mentioned previously, the serine synthesis promoting enzyme PHGDH is commonly upregulated in cancer via copy number gain or as a result of oncogenic ATF4, NRF2 and hypoxic signaling (126,168,403–405). PHGDH inhibitors have been developed and shown to successfully suppress serine synthesis and cancer cell proliferation *in vitro* and *in vivo* (406–408), however they display poor cancer selectivity and affect the central nervous system (409,410).

More promising proof-of-concept studies focusing on targeting SHMT1 and MTHFD2 have demonstrated great potential and selective anticancer efficacy in *in vivo* models (181,210,222,230), yet have also indicated that a complete inhibition of one-carbon metabolism may only be achieved by concomitant targeting of multiple enzymes. Thanks to its cancer-enriched profile, MTHFD2 remains an optimal candidate for development of inhibitors (207). A comprehensive comparison of MTHFD1, MTHFD2 and MTHFD2L crystal structures and homology models (401,411), together with our group's recent determination of MTHFD2 crystal structure (412), have made great strides towards reaching this goal. While the cofactor binding site seems to be highly conserved between these enzymes and among other NAD/NADP-binding proteins, and is thus unsuitable for inhibitor development, the substrate binding pocket contains a few residues which vary between MTHFD1 and MTHFD2, and could prove useful to achieve selectivity. Although the recent approval of personalized therapies targeting metabolic mutations such as IDH inhibitors has caused much excitement and renewed hopes, resistance has already been described through additional mutation of the target (413).

Taken together, these efforts to target metabolic pathways expose the need for complementary combinatorial strategies to prevent resistance and improve efficacy, and highlight the need to widen our therapeutic repertoire to identify optimal combinations for specific tumor contexts.

2 DOCTORAL THESIS

2.1 RESEARCH AIMS

In recent years, both PFKFB3 and MTHFD2 have been extensively validated as attractive anticancer targets, due to their overexpression in tumors, their various described roles supporting cancer progression and their negative association to disease prognosis (101,207,210,401,414).

PFKFB3 is a vital regulator of glycolysis with the highest kinase-to-phosphatase activity ratio among the PFKFB isoforms, which promotes high glycolytic rates (60). Exhaustive studies have shown that cancer cell growth, proliferation and metastasis are stimulated when PFKFB3 is overexpressed or activated (80,414). More recently, researchers have linked glycolysis and PFKFB3 to DNA repair mechanisms, which implied yet another role for PFKFB3 in supporting genome stability (124,125).

MTHFD2 is canonically an enzyme of mitochondrial one-carbon metabolism; an oncofetal protein normally expressed only during embryogenesis, yet commonly reactivated in cancer cells (386). MTHFD2 has also been found to localize to the nucleus in proximity to DNA replication sites (211). Analysis of publicly available gene expression datasets revealed that MTHFD2 is overexpressed in cancer patients resistant to radiotherapy (415), with data from our group showing that depletion of MTHFD2 in cancer cells results in DNA damage, suggesting a more direct role for MTHFD2 in genome maintenance.

New therapeutic strategies are needed to improve the prognosis of cancer patients. Based on our preliminary data, we hypothesized that both PFKFB3 and MTHFD2 could also support proliferation and cancer cell survival via novel functions linked to DNA repair and genome stability. Therefore, the specific aims of this thesis were designed to

(1) characterize the roles of PFKFB3 and MTHFD2 in nucleotide metabolism, DNA replication and HR in cancer cells, and

(2) develop, validate and benchmark potent inhibitors for therapeutically targeting PFKFB3 and MTHFD2 in neoplastic malignancies.

These aims were addressed in the component papers of this thesis and guided the outstanding research questions posed in each of the studies as follows:

Paper I

• Does PFKFB3 support DNA synthesis only through its effect on glycolysis, or does it have a more direct role?

• Does the genome stability supporting function of PFKFB3 require its enzymatic activity?

• How does PFKFB3 interact with DNA synthesis and repair enzymes to support this role?

• Can the development of more potent and selective PFKFB3 inhibitors aid in uncovering new target biology? Does PFKFB3 inhibition phenocopy the depletion of PFKFB3 by RNAi?

• Can PFKFB3 inhibition be used to sensitize cancer cells to therapies targeting DNA stability?

Paper II

• Can the co-crystal structure of LY345899 in the MTHFD2 substrate-binding site reveal new insights over existing homology models to guide drug development?

• Is there an effect of co-factor binding on substrate affinity and enzyme activity? How does co-factor preference relate to the physiological subcellular location of MTHFD2?

• What differences, if any, between MTHFD1 and MTHFD2 could be exploited to develop isozyme selective inhibitors?

Paper III

• Is MTHFD2 enzymatic activity needed for its role in genome stability? What stage of the DNA life cycle is MTHFD2 crucial for?

• Can specific metabolites rescue the phenotype upon loss of MTHFD2, and can they hint at the pathway deregulations causing the observed genetic instability?

• How can structure-activity relationship models of MTHFD2 improve the design of our first-in-class substrate-based inhibitors?

• Can potent MTHFD2 inhibitors identified by biochemical, cellular and target engagement profiling confirm the emerging role of MTHFD2 in replication stress? Do the results reflect biochemical potency rankings, and can these inhibitors be used to reveal new target biology?

• What is the cell killing mechanism behind MTHFD2 inhibitors? Does this phenotype follow the target's cancer-enriched expression profile?

• How can we exploit these mechanistic insights to devise rational therapy combinations?

• Do MTHFD2 inhibitors display *in vivo* antitumor efficacy? How do they compare to standard of care compounds?

Paper IV

• Is MTHFD2 involved in other genomic maintenance processes besides replication?

• How is MTHFD2 modulated upon DSB DNA damage?

• Can depletion of MTHFD2 potentiate IR-induced DNA damage?

• Where in the DDR cascade does MTHFD2 come in?

• Can MTHFD2 inhibitors mimic HR deficiency and sensitize cancer cells to PARP inhibition?

2.2 METHODOLOGY

The methods and approaches used throughout the studies comprised in this doctoral thesis were highly multidisciplinary and strengthened our research findings from different technical perspectives, including structural biology, biochemistry, medicinal chemistry, molecular and cell biology, as well as translational pharmacology.

Detailed descriptions of all the techniques used in this doctoral project can be found in the individual study publications and manuscripts. Therefore, only key research-area specific methods and considerations are discussed below.

2.2.1 Cellular thermal shift assay (CETSA)

The ability to study protein-inhibitor interactions under physiological conditions is a valuable and integral part of drug development, not only to directly confirm target engagement, but also as a tool to deconvolute target biology.

Thermal shift assays (TSAs) are used to measure changes in the thermal stability of proteins against denaturation in response to different conditions, such as ligand concentration, pH, sequence mutations, etc. The thermal unfolding of a protein can be followed over a temperature gradient using differential scanning fluorometry (DSF) (416), producing a melting curve from which the protein's melting temperature (Tm) can be determined. It has long been established that ligand binding modulates the thermal stability of proteins (Koshland, 1958; Kranz and Schalk-Hihi, 2011), either destabilizing it or more often by increasing its thermal stability, resulting in a measurable shift of the melting temperature (ΔTm).

The cellular thermal shift assay (CETSA) was developed to assess protein thermal stability changes directly in living cells, whereby whole cells or cell lysates, and not just purified proteins, are incubated with a ligand and subjected to a thermal gradient to assess target protein unfolding in its native environment (417). The idea is that ligand-stabilized proteins will resist denaturation and stay in solution while unbound proteins will aggregate and precipitate. Subsequent quantification of the soluble protein fraction by Western blot, amplified luminescent proximity homogeneous assays (e.g., AlphaScreen®) or mass spectrometry can determine the extent to which ligand-binding affects native protein unfolding (**Figure 10**).

This method has several advantages over traditional TSA methods, namely that it requires no ligand modification (e.g., linker attachment), no cloning work to introduce reporter tags on the protein, and no specialized equipment besides a polymerase chain reaction (PCR) machine, a table-top centrifuge and high affinity antibodies against the target (418). As a label-free method, CETSA can be applied to a wide range of cells including *in vitro* cells from culture, *ex vivo* and *in vivo* xenograft material, as well as primary tissue samples from patients. It can be adapted to automated and high-throughput formats, thus making it suitable to directly screen large compound libraries.

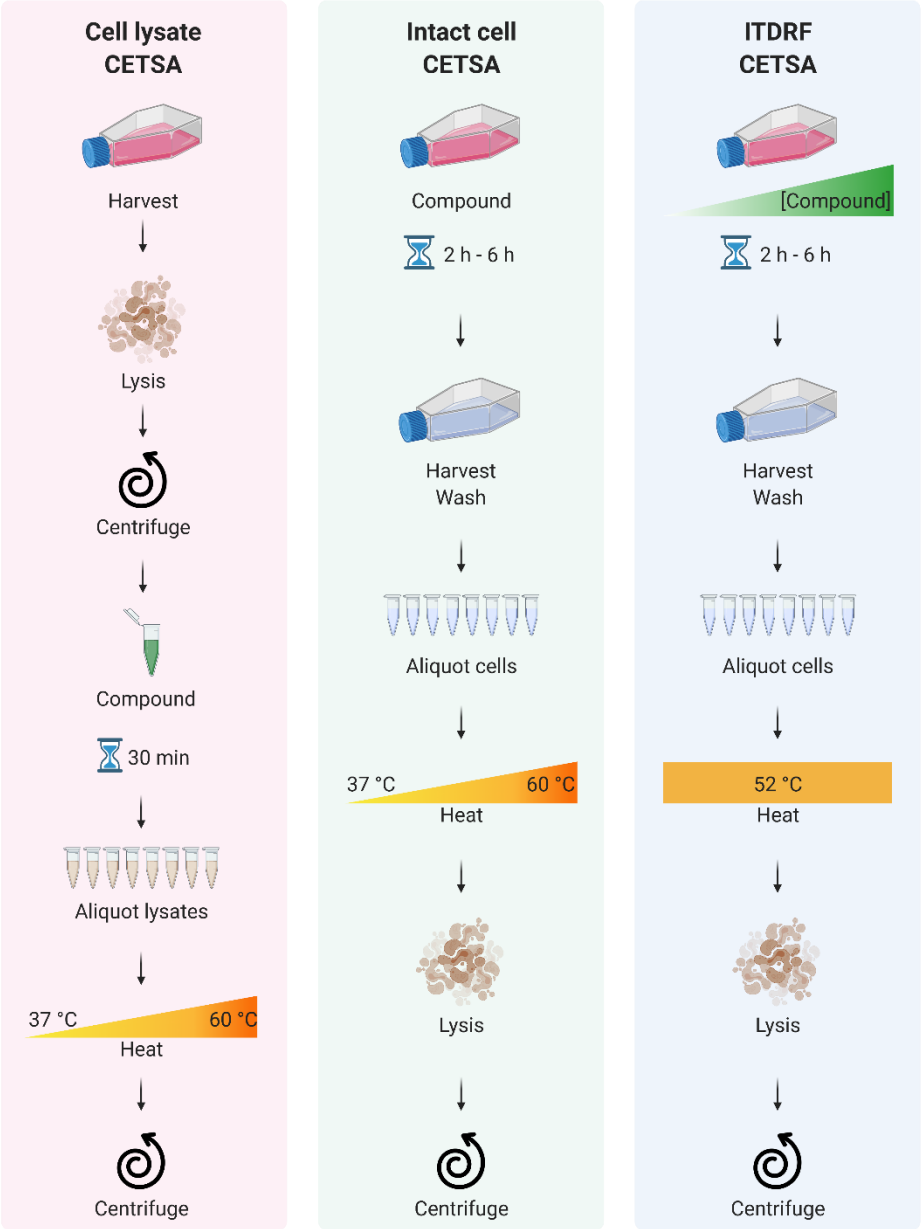

Figure 10. Sample preparation for CETSA experiments. ITDRF: isothermal dose-response fingerprint. Briefly, cells or cell lysates were incubated with test compounds or vehicle controls, at a single dose or a dose-range, then aliquoted and heated to different temperatures, and finally centrifuged to separate the aggregates from the soluble protein fraction. All buffers were complemented with protease inhibitors to minimize protein degradation during sample preparation. Created with BioRender.com

Despite its many virtues, some limitations still remain, in particular pertaining to its target- and ligand-specific optimization as well as the detection and accurate quantification of stabilized proteins. While high affinity antibodies against well-known proteins are readily available, newer and perhaps understudied targets might not have suitable antibodies to reliably make use this method (418). One workaround could involve tagging proteins of interest with reporter groups, at the risk of dramatically reducing the number of cells in which the assay could be performed. Unbiased methods such as quantitative mass spectrometry are increasingly being used to assess target protein stabilization, even when the putative targets are unknown *a priori*, however this incurs significant cost and effort (419,420).

Although the technique has a high degree of reproducibility and false positives are uncommon, it is possible for a compound to bind proteins in the same pathway or in the same protein complex as the intended target and manifest as direct target engagement even if it does so via indirect effects. Complementary use of intact cell and cell lysate CETSA may contribute additional layers of information regarding indirect effects on protein complexes as they are disrupted in lysate conditions, as well as help determine compound uptake or membrane permeability properties.

2.2.2 Direct repeat green fluorescent protein (DR-GFP) assay

To investigate molecular deregulations affecting the fidelity and efficiency of DSB repair, various GFP-based reporter systems have been specifically developed to study and differentiate between defined repair outcomes such as HR, NHEJ and single strand annealing (SSA). These reporters all use rare-cutting endonucleases, mainly I-SceI, to induce DSBs and then monitor their repair (421).

Among the most commonly used tools to study DSB-induced HR activity is one such reporter system, DR-GFP, which measures GFP fluorescence as a readout of HR capacity (**Figure 11**) (422). The reporter plasmid contains a full-length GFP sequence with an I-SceI cleavage site affecting the open reading frame (ORF), as well as an additional GFP gene fragment with the correct ORF sequence, which cannot produce functional GFP protein but serves as a template to restore the upstream full-length sequence via gene conversion. In cells which stably express the reporter plasmid, the GFP readout is measured by transiently transfecting I-SceI and allowing 2-3 days for the cells to complete the repair, depending on the cell type (421). The GFP readout is commonly measured by flow cytometry, but can also be quantified by high-throughput immunofluorescence microscopy or even by PCR amplification of repair products using primers flanking the I-SceI cleavage sites (421). Recent developments in CRISPR/Cas9 gene editing technology have taken these assays to the next level, allowing the precise introduction of SSBs and DSBs at desired loci to study their repair (423).

One of the main limitations of the assay is the low frequency at which these cleavage and repair events occur, which result in the amount of GFP+ cells after DSB induction ranging between 1% and 5%. Furthermore, since the frequency of DSB repair events is directly dependent on I-SceI transfection efficiency and cell survival, ways to standardize or control for these parameters may be required, especially when comparing results between different cell lines. An example of such a control would be to perform parallel co-transfection of I-SceI with a second vector containing mCherry or DsRed, or use I-SceI vectors in which the I-SceI protein is tagged with a reporter marker such as hemagglutinin (HA) which can be used to measure expression levels by Western blot with anti-HA antibodies (424). In our experiments, we believe such controls were not strictly necessary as we only used U2OS cells, which showed little variation in transfection efficiency between replicates, and always prepared transfection master mixes with common reagent batches.

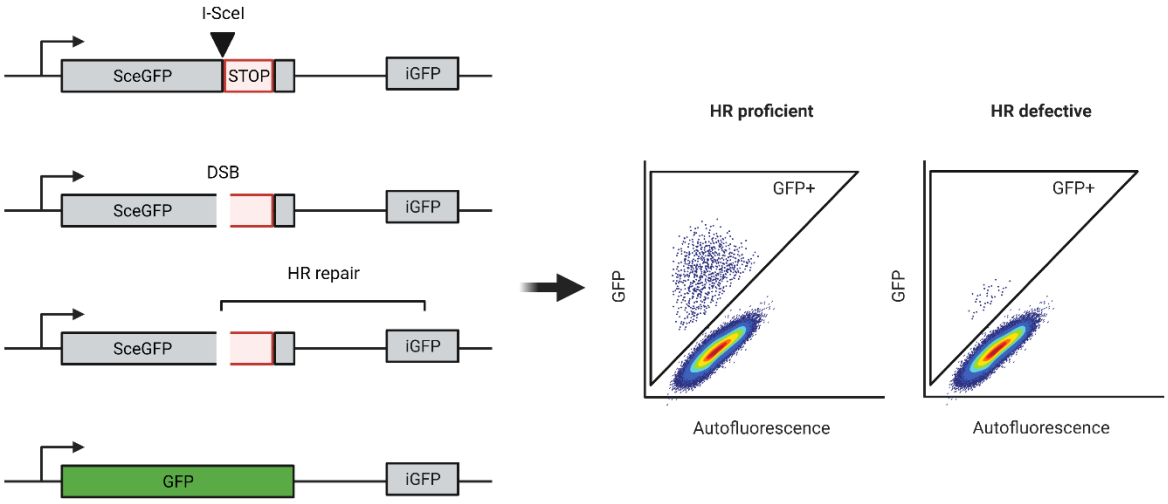

Figure 11. Graphical representation of the direct repeat GFP (DR-GFP) reporter assay. The reporter plasmid contains a modified GFP sequence containing an I-SceI site and in-frame stop codons (SceGFP), together with an internal truncated GFP fragment (iGFP). The cells can use the iGFP as template to repair the endonuclease-induced DSB by HR gene conversion, resulting in a functional GFP gene which can be used to quantify HR-proficient cells by flow cytometry or immunofluorescence microscopy. Created with BioRender.com

Another consideration is the tradeoff between increasing transfection efficiency and toxicity. To optimize the number of cells seeded for maximal transection efficiency while ensuring enough cells available for flow cytometry after 3 days without reaching confluency, we opted for a low-throughput 6-well format. While this allowed us to harvest enough cells for proper statistical analysis of the low frequency events, it came at the cost of higher usage of expensive transfection reagents.

2.2.3 DNA fiber assay

Understanding replication stress response mechanisms and perturbations has particularly important implications for genetic instability in the context of cancer establishment and progression (279,379). The DNA fiber assay is one of the most powerful and widely used tools to study perturbations of replication dynamics at the single-molecule level. This technique relies on cells being able to incorporate nucleotide analogs into nascent DNA and is commonly used to evaluate how genotoxic agents affect DNA replication (425,426).

In short, dividing cells are sequentially pulse labeled with two different halogenated pyrimidine nucleoside analogs, which get readily incorporated into replicating DNA (427). The nucleoside analogs 5-chloro-2′-deoxyuridine (CldU) and 5-iodo-2′-deoxyuridine (IdU) are commonly used and are added directly to the culture medium. After incubation with the first analog (e.g., CldU), the cells are carefully washed with pre-warmed medium, and incubated with the second analog (e.g., IdU). The concentration of the second analog should be approximately 10-fold higher than the concentration of the first analog to ensure that the second analog is able to displace any remaining initial analog that was not removed during the washing steps. Labeling

and washing steps should be performed as quickly and gently as possible, to prevent major disruption of ongoing replication. After labeling, cells are harvested and lysed, with DNA being spread or combed onto microscope slides, then fixed and immunostained with anti-CldU and anti-IdU antibodies (**Figure 12**). Imaging of the DNA fibers is performed by fluorescence or confocal microscopy, where special attention should be put into imaging only the regions where the fibers are well separated and not entangled, and to capture images in different areas along the slide as only one region may not provide representative data. Finally, fiber track length is manually scored using ImageJ or automatically by software algorithms, depending on the uniformity of the DNA fibers. A minimum of 100 fibers per condition should be quantified to gain a robust understanding of the overall effects on replication.

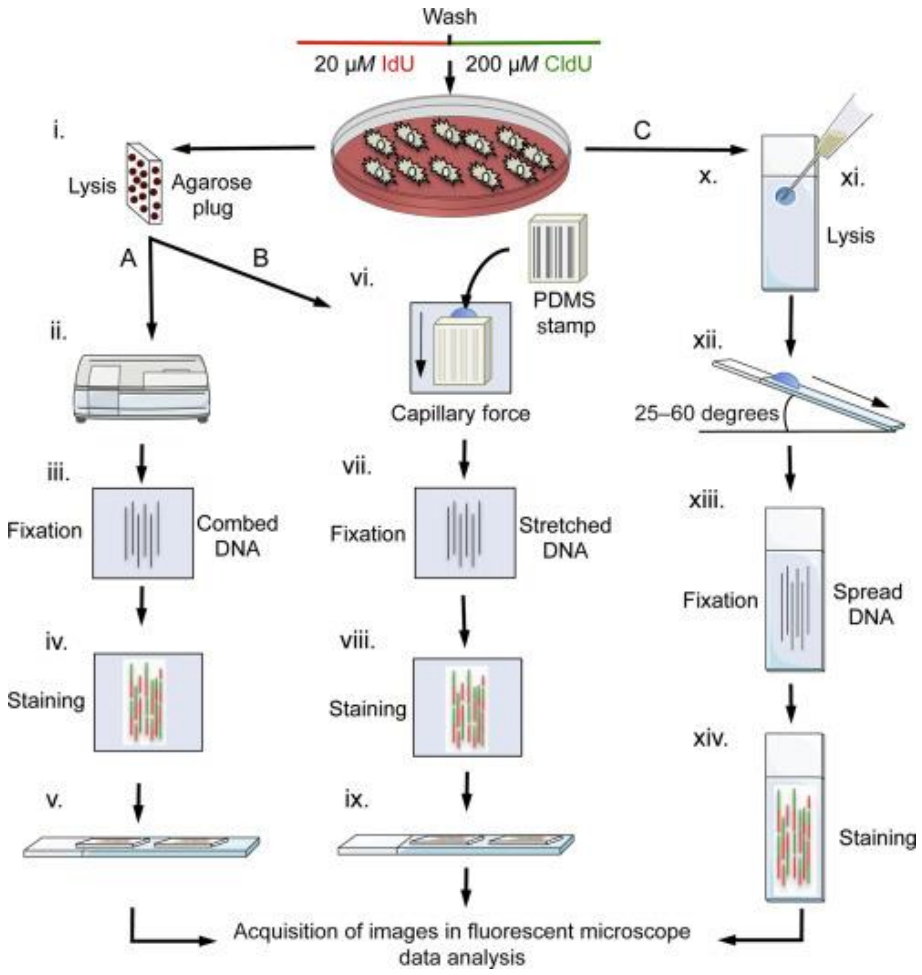

Figure 12. Schematic of the three main methods to prepare DNA fibers. (A) DNA combing: cells are resuspended and lysed in an agarose plug, then DNA is combed with a combing machine onto a silanized coverslip. (B) Microfluidic-assisted replication tract analysis (maRTA): cells are embedded and lysed in an agarose plug, then DNA is stretched using a polydimethylsiloxane (PDMS) patch containing small capillaries. A drop of isolated DNA is added at one of the capillary ends of the PDMS patch, which stretches the DNA onto the silanized coverslip using capillary force. (C) DNA spreading: a drop of prelabeled cells is transferred to a positively coated microscope slide and lysed. The slide is then tilted at a 25–60 degrees angle to allow DNA spreading down the slide. Following spreading by any of these methods, DNA is fixed and immunostained. DNA fiber images can then be acquired using a fluorescent microscope. Reprinted from Quinet *et al.*, 2017 (425),with permission from Elsevier.

The order and duration of the analog labeling incubations depends on the biological question being addressed. The DNA fibers assay is highly versatile in the variety of replication parameters it can assess. The original scheme whereby both analogs are added for the same amount of time can identify elongating forks, stalled/collapsed forks, initiation events (origin firing) and replication collision terminations (**Figure 13**) (425).

One of the most important readouts of this assay is the estimation of replication fork velocity. After measuring the length of labeled tracts, the values are converted from micrometers (μm) into kilobases (kb). For fibers obtained by combing the conversion factor is 2 kb/μm (428), while for fibers obtained by spreading, like the ones used throughout this thesis project, the conventionally accepted conversion factor is 2.59 kb/μm (429,430). Fork speed can then be calculated by dividing the length of the tract by the labeling time (kb/min). Fork velocity can vary from 0.5 to 3 kb/min depending on the particular organism and cell type (427,430–433).

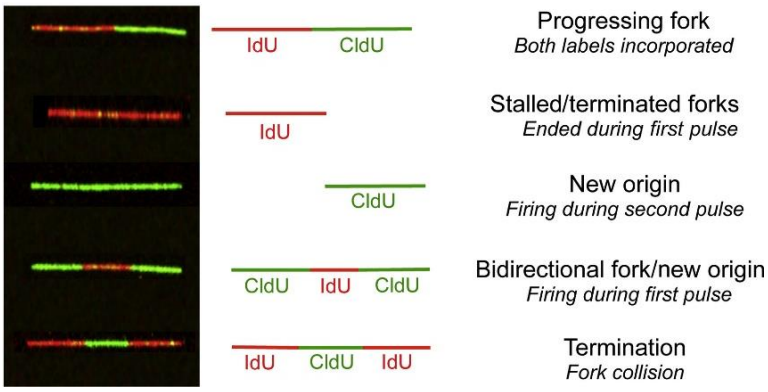

Figure 13. Different replication events that can be studied by DNA fiber analysis. Replication parameters observed by DNA fiber assay and their interpretations. Reprinted from Quinet *et al*., 2017 (425), with permission from Elsevier. Copyright © 2017 Elsevier Inc. All rights reserved.

One of the main limitations of the DNA fiber technique is that it is delicate and time consuming, allowing preparation of only a handful of samples at a time. Compared to DNA combing and stretching, DNA spreading requires the least material and equipment, however it still takes two full days for sample preparation. Delicate handling of the DNA fibers is paramount to minimize breakage. Moreover, spreading DNA using gravity can lead to non-uniform DNA fibers with increased risk of fiber entanglement, reducing the number of fibers available for quantification. In our studies we were able to improve DNA protection, facilitate fiber elongation and reduce reagent volumes by switching from conventional microscope slides to multichannel slides.

Another drawback of the DNA fiber technology is that it cannot detect ssDNA regions which are important intermediates in replication stress mechanisms, such as nuclease resection of stalled forks required for restart. The resolution of this assay cannot distinguish these < 1 kb regions, requiring more powerful methods like electron microscopy to visualize them (434).

2.2.4 Uracil DNA glycosylase (UNG) modified alkaline comet assay

The comet assay, or single-cell gel electrophoresis, is a straightforward and sensitive method for measuring DNA strand breaks in cells independent of organism or tissue origin, with numerous variations generated since it was first described in 1984 to assess different types of damage and repair (435,436). A notable modification was the "alkaline" comet assay which uses a higher denaturation pH to convert a wider range of DNA lesions into breaks (e.g., alkali-labile sites such as alkylated or apurinic/apyrimidinic (AP) sites), thereby making them easier to detect (437). Due to its increased sensitivity over the original comet assay, the alkaline version is usually preferred.

The principle behind the technique is the ability of DNA to form supercoiled loops called nucleoids when stripped of protein associations (438). Intact DNA retains a compact structure while DNA breaks induce a loss of supercoiling and relaxation of the nucleoid structure, which can be measured using electrophoresis. To achieve this, cells are suspended in low-melting-point (LMP) agarose at 37 °C and embedded onto microscopy slides, then lysed with detergent and high salt to disrupt DNA-protein binding. In the alkaline version of the assay, nucleoid DNA is then denatured in high pH conditions (pH > 13) and subjected to an electric field allowing uncoiled and fragmented DNA to migrate away from the main bulk of the nucleoid towards the anode. This creates comet-like structures, which are then stained with a fluorescent DNA dye for microscope visualization and image acquisition (**Figure 14**).

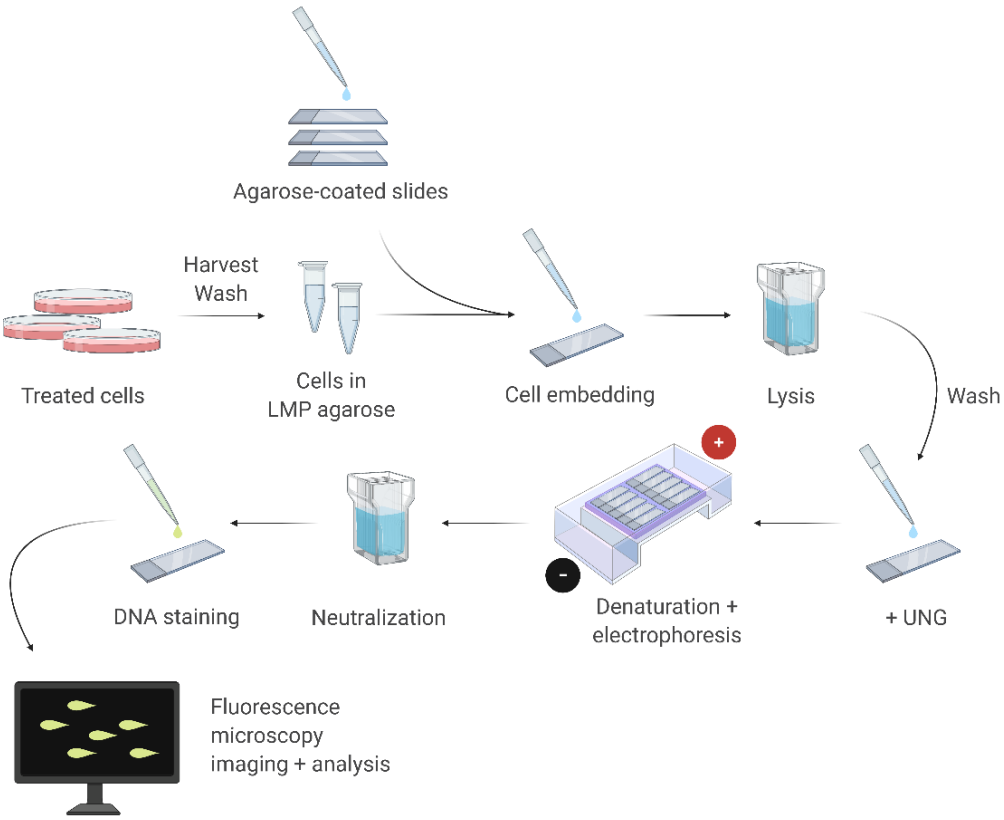

Figure 14. Graphical description of the UNG modified alkaline comet assay protocol. From the moment the cells are embedded in the agarose, all assay steps are performed in the dark to minimize photodegradation of DNA. Created with BioRender.com

The intensity of the comet tail compared to the head is an indication of the number of DNA breaks. This is measured either as tail DNA percentage or tail moment, defined as the product of the tail length and tail DNA percent (439). The sensitivity of the assay ranges from approximately 50 to several thousand breaks per cell, which covers low endogenous DNA stress levels as well as high DNA damage levels caused by genotoxic drugs (440,441).

An important modification of the assay has been the addition of an enzymatic digestion step, where the nucleoids are incubated with different DNA repair nucleases to measure specific DNA lesions. For example, DNA glycosylases such as formamidopyrimidine DNA glycosylase (FPG), 8-oxoguanine DNA glycosylase (OGG1) and uracil-DNA glycosylase (UNG) are commonly used in comet assays to study oxidative DNA damage and uracil misincorporation (442–444). Although the main utility of this method is to measure DNA breaks, several modifications to the assay have enhanced its power to distinguish between SSBs and DSBs (e.g., 2-dimesional two-tailed TT-comet (445)) and even evaluate DNA repair mechanisms at specific gene regions (e.g., Comet-FISH (446)).

Despite its relative simplicity and versatility, the main limitations of the comet assay remain how delicate, low throughput and labor-intensive it is. From cell culture to slide storage and image acquisition, every step should be carefully executed to avoid high background DNA damage levels, including working in the dark for several hours at a time. Cells must be low passage, as cells cultured for more than 12-15 passages tend to display higher basal DNA breaks that could mask treatment effects. Agarose embedding of the cells is another delicate step, as gel temperature should be carefully monitored to prevent additional damage. In between incubation steps and during storage coverslips are used to prevent drying of the agarose, however improper removal of the coverslip can often result in gel break or detachment. Electrophoresis tank space is yet another limiting factor, particularly when experimental conditions tend to exponentially increase with the addition of enzymatic digestion steps, varying drug concentrations, different timepoints, technical replicates and relevant controls.

Variability in the comet assay represents another important issue. While intra-laboratory reproducibility is typically high, inconsistencies between research groups are common and likely due to discrepancies in protocol incubation times, staining, analysis, and other experimental parameters (440,447). Comet scoring can also be time-consuming and potentially biased if automated software is not employed. Although various high throughput modifications have been developed in recent years, scoring remains a prominent bottleneck (448).

2.2.5 Ethical implications

Most of the work presented in this project has been carried out using established cancer and non-transformed cell lines, for which no ethical permit is required. The *in vivo* studies to test MTHFD2 inhibitors in xenograft mouse models were performed by trained professionals in accordance with ethical permits N217/15 and N89/14.

2.3 SUMMARY OF RESULTS

2.3.1 Paper I: Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination

Prior to this study, PFKFB3 had been validated as a promising target to exploit glycolytic deregulation in cancer (61,65,77,110,449). Indications of its non-canonical functions in the nucleus and on p53-mediated repair of UV lesions (90,124), together with the identification of PFKFB3 in genome-wide siRNA screens to detect DDR factors and the observation that PFKFB3 was upregulated in radiotherapy-resistant patients (415,450,451), prompted us to investigate its potential involvement in the repair of IR-induced DSBs. In this study, we reported a novel role for PFKFB3 in HR repair of DSBs in cancer cells and described the development of a new PFKFB3 inhibitor, which we used to validate our biological findings.

First, we assessed whether PFKFB3 was recruited to DSB sites and co-localized with DNA damage marker γH2AX upon IR using *in situ* cell fractionation and confocal microscopy. We found that ATM activity, but not ATR or DNA-PK, mediated PFKFB3 recruitment to DSB sites. Depletion of γH2AX and MDC1 by siRNA also reduced PFKFB3 co-localization to IR-induced foci (IRIF). Furthermore, we observed that PFKFB3 foci co-localized with important DSB repair factors, and that depletion of PFKFB3 abolished recruitment of HR factors BRCA1, RPA32 and RAD51 to damage sites. To confirm functional engagement of PFKFB3 in HR we performed DR-GFP assay in PFKFB3-silenced cells, showing that loss of PFKFB3 activity reduced HR potential by 60% in a manner unexplained by changes in cell cycle distribution. Clonogenic survival assays were performed to determine the effects of PFKFB3 depletion in combination with IR on cancer progression, demonstrating that concomitant PFKFB3 silencing significantly enhanced radiosensitivity by approximately 6-fold compared to cells treated only with IR.

In an attempt to pharmacologically recapitulate the HR effects observed with siRNA depletion, we used an established inhibitor of PFKFB3, 3PO. Nevertheless, we could not confirm inhibition of PFKFB3 with this compound, nor could we validate the HR effects observed with siRNA. Therefore, we established a collaboration with the pharmaceutical company Kancera to develop alternative inhibitors, starting with a drug screening campaign of over 50,000 compounds which yielded over 100 hits with low micromolar to nanomolar IC_{50} values against PFKFB3. Non-ATP competitive hits were progressed along the pipeline and evaluated for selectivity against the other PFKFB isozymes, resulting in a class of phenylsulfonamido-salicylic acids which effectively reduced intracellular F2,6BP levels and cell viability in different cancer cells. In parallel, assessment of biophysical and ADME parameters to confirm pharmacological suitability of the compounds revealed KAN0438241 and its prodrug KAN0438757 (KAN757) as potent and selective representatives of this phenylsulfonamido-salicylic acid family of PFKFB3 inhibitors. In cells, KAN757 was shown to bind and stabilize PFKFB3 using CETSA, with prolonged stability and inhibitory capacity lasting at least 72 h, corresponding to the duration of most cellular assays. Finally, selectivity over other kinases

beyond the PFKFB isozymes was evaluated, with no significant inhibition being observed for KAN0438241 or KAN757 across a panel of 97 distinct kinases.

To assess whether the HR-supporting role of PFKFB3 was mediated by its kinase activity, we repeated some of the key siRNA experiments with KAN757. We found that KAN757 phenocopied the results observed upon PFKFB3 depletion, namely the impaired recruitment of RPA32 and RAD51 to IR-induced DSB sites downstream of MRN signaling and the significant reduction of HR potential in DR-GFP assays. Dose-dependent radiosensitization by KAN757 was also observed in cancer cells but not in non-transformed cells.

We hypothesized that PFKFB3 could promote HR repair via effects on nucleotide metabolism, which we confirmed by monitoring 5-ethynyl-2′-deoxyuridine (EdU) incorporation into DNA following IR-induced damage in the presence of KAN757. Nucleotide production at sites of DNA synthesis is mediated by RNR subunit RRM2 (274,288), thus we investigated whether PFKFB3 activity affected RRM2 recruitment upon DSB formation. Knockdown and inhibition of PKFBF3 by KAN757 prevented RRM2 recruitment to DSB sites to a similar extent than ATM inhibition, depletion of RRM2 resulted in a similar decrease of HR activity in DR-GFP assays as depletion or inhibition of PFKFB3, and finally a direct interaction between PFKFB3 and RRM2 could be observed via co-immunoprecipitation, altogether suggesting PFKFB3 could support HR repair by facilitating local supply of dNTPs. Next, we measured cellular dNTP levels upon KAN757 inhibition of PFKFB3, showing significantly reduced nucleotide concentrations equally across purine and pyrimidine pools. Moreover, effects of KAN757 on DNA replication speed and viability could be rescued by supplementation with nucleosides.

Taken together, the work presented in this study demonstrated a new HR-promoting role for nuclear PFKFB3 regulating local dNTP production via recruitment of RRM2 in response to MRN-ATM-γH2AX-MDC1 signaling upon IR-induced DSBs. This study also presented the development of a potent, selective and cell active PFKFB3 inhibitor KAN757, and validated it as a radiosensitizer with potential implications for the future improvement of cancer therapy.

2.3.2 Paper II: Crystal structure of the emerging cancer target MTHFD2 in complex with a substrate-based inhibitor

At the start of this study, MTHFD2 had recently reemerged as a promising anticancer target indispensable for cancer cell proliferation, despite having been detected in rapidly proliferating embryonic tissue and tumor cells in 1985 (48,176,207,401). In the years since, no MTHFD2 inhibitors had been described, and attempts to determine the protein's 3D strcuture had thus far relied on sequence homology models based on the structure of the MTHFD1 dehydrogenase-cyclohydrolase (DC) domain (178,411) and MTHFD2 homologs in bacteria and yeast (452,453). In this study we described the first inhibitor to be identified for human MTHFD2, and with it, the first crystal structure of MTHFD2. This work provided an important piece of structural foundation for drug discovery efforts to be built upon to rationally design potent and selective MTHFD2 inhibitors for cancer treatment.

We started by expressing and purifying human MTHFD2 and MTHFD1-DC domain in *E. coli*, and used the commercial NAD(P)H-Glo assay (Promega) to measure their enzymatic activity. We then tested a known MTHFD1-DC ligand, LY345899, and found that it also substantially inhibited MTHFD2 enzymatic activity with an IC_{50} value of 663 nM. Target engagement of MTHFD2 by LY345899 was first evaluated by DSF, showing strong stabilization of the protein with an associated ΔTm of 11 °C. This was then confirmed in cellular lysates with CETSA and the drug affinity responsive target stability assay (DARTS), based on the same principle as CETSA, but rather than subject the lysates to thermal degradation, the stability of ligand-bound proteins is measured in response to protease degradation. Paradoxically, LY345899 did not affect cancer cell viability and CETSA in intact cells with LY345899 did not result in MTHFD2 stabilization, suggesting the compound is poorly permeable.

High quality crystals of MTHFD2 bound to LY345899 in the presence of cofactors NAD+ and inorganic phosphate (Pi) were produced and used to determine the structure of MTHFD2 by X-ray diffraction at the European Synchrotron Radiation Facility to a resolution of 1.9 Å. The overall homodimeric structure coincided largely with previous models, but identified important differences relative to MTHFD1-DC structure that could prove useful to design isozyme selective inhibitors. The co-factor binding sites correlated well to known cofactor preference (i.e., why MTHFD2 preferentially uses NAD+ and Pi over NADP+), as well as previous observations of site-mutants and their effects on enzyme activity. The interactions between LY345899 and the substrate binding site revealed various insights on ligand binding modality and identified the key residues responsible for substrate binding and enzyme activity. The difference in affinity to LY345899 between MTHFD1-DC and MTHFD2 was also reflected by the presence of important ligand-binding residues in MTHFD1 which are not conserved in MTHFD2. Altogether, these observations provided a sound starting point towards the advancement of other substrate-based inhibitors optimized towards MTHFD2 and the development of robust structure-activity relationship (SAR) models.

2.3.3 Paper III: Targeting MTHFD2 kills cancer via thymineless-induced replication stress

From the literature we found that, while MTHFD2 had been extensively validated in different *in vitro* and AML *in vivo* models as being crucial for tumor cell proliferation and survival (207,210,401), no study had directly assessed whether the enzymatic activity of MTHFD2 was necessary for its role in cancer progression. With the structural insights from **Paper II**, we devised point mutations in the substrate-binding pocket of MTHFD2 to abolish its enzymatic activity, and performed viability rescue experiments where endogenous MTHFD2 levels were depleted and either wild-type or catalytically dead constructs were expressed. We found that mutations in the substrate-binding site affected the protein's ability to rescue the viability phenotype. Furthermore, we noticed that depletion of MTHFD2 resulted in increased levels of DNA damage marker γH2AX, which could also be restored by overexpression of the wild-type

but not the catalytically dead mutant, highlighting the importance of MTHFD2 activity for its role in cancer.

Additional metabolic rescue experiments revealed that the decrease in viability upon MTHFD2 depletion could be rescued by supplementation of either nucleosides, thymidine and folate, but not glycine or hypoxanthine, suggesting that production of CH_2-THF by MTHFD2 required for *de novo* thymidylate synthesis is key to supporting cancer cell proliferation. Analysis of γH2AX levels across the cell cycle showed that DNA damage upon loss of MTHFD2 accumulated in S phase, indicative of replication stress and consistent with its association to replication sites in the nucleus (211). These observations were confirmed by DNA fiber assays showing significantly impaired replication fork speeds in MTHFD2-depleted cells.

The relatively low potency and poor permeability of LY345899 made it unsuitable to study the pharmacological inhibition of MTHFD2 in cells, which encouraged us to develop more potent and cell-active MTHFD2 inhibitors. In search of promising compounds for lead optimization we screened over 500,000 molecules but found no suitable candidates, which redirected us towards a substrate-based drug development approach capitalizing on the structural understanding gained from **Paper II**. Successive rounds of chemical design, synthesis and evaluation of biochemical and cellular activity yielded a well-refined SAR model and a series of substrate-like compounds with significantly improved potency and cell activity compared to LY345899. Three representative MTHFD2 inhibitors (MTHFD2i) in this series, TH7299, TH9028 and TH9619, were selected for further biological evaluation and as tools to investigate MTHFD2 mechanisms of action. These compounds were confirmed to bind and stabilize MTHFD2 using DSF, surface plasmon resonance (SPR), CETSA and DARTS assays, while close pathway targets such as MTHFD1L, DHFR, TS, SHMT1 and SHMT2 remained unaffected in activity and target engagement assays. Although related isozymes MTHFD1 and MTHFD2L were also found to be inhibited by MTHFD2i, CRISPR/Cas9 MTHFD2 knockout cells were shown to be orders of magnitude more resistant to TH9619 than MTHFD2 wild-type cells, providing strong evidence that MTHFD2i-induced cancer cell toxicity is largely mediated by on-target MTHFD2 inhibition.

In a panel of hematological cells including ALL and AML cell lines as well as non-transformed lymphoblastoid LCL cells, MTHFD2i displayed particular efficacy in AML cell lines and the most cancer-specific profile, compared to the standard of care compound cytarabine (AraC) and the antifolate methotrexate which also affected LCL cell viability. Relative to the AML-specific differentiation agent all-*trans* retinoic acid (ATRA), MTHFD2i also induced myeloid differentiation, while their anticancer efficacy was extended beyond AML to T-ALL Jurkat cells, expanding the potential therapeutic applications of these compounds. The cancer-selective profile of MTHFD2i was confirmed in CCD 841 normal colon epithelial cells and CD34+ bone marrow cells from healthy donors, where MTHFD2i had little effect on viability compared to a variety of anticancer agents.

Next, we investigated whether MTHFD2i induced replication stress in cancer cells similar to MTHFD2 depletion. We observed that MTHFD2i induced accumulation of DNA damage in S

phase, impaired replication fork speeds, led to replication fork collapse and activated intra-S phase checkpoint pro-apoptotic signaling. Combination of MTHFD2i with replication stress response blockers (e.g., inhibitors of ATR, CHK1, WEE1) synergized to reduce cancer cell viability and induce apoptosis. Moreover, we showed that MTHFD2i introduced replication stress selectively in transformed cells, while methotrexate and ATR inhibitor affected replication fork progression indiscriminately in both normal and transformed cells. In line with RNAi data, viability, replication stress and differentiation phenotypes induced by MTHFD2i could be completely rescued by addition of thymidine, in contrast to established antifolates and ATRA, suggesting a distinct mechanism of action for MTHFD2i via induction of thymineless death selectively in cancer cells.

A consequence of thymidylate depletion is an increased frequency of dUTP misincorporation into DNA, which causes replication stress and is deleterious for cells. Hence, we evaluated whether MTHFD2i affected genomic uracil misincorporation. Using the modified alkaline UNG comet assay and LC-MS measurements of genomic uracil, we confirmed that MTHFD2i induced significantly higher levels of uracil in DNA than TS inhibitor 5-FU and standard antifolates, and that this effect could also be reversed by thymidine supplementation. Inhibition of dUTPase promotes the accumulation of dUTP by preventing its degradation; consequently, MTHFD2i and dUTPase inhibition synergized to potentiate uracil incorporation into DNA, induce dose-dependent reduction of cancer cell viability and increase apoptosis.

Evaluation of clinically relevant pharmacological parameters revealed TH9619 to be a promising candidate for further translational applications, as it displayed good solubility and stability, low plasma protein binding, and had clean selectivity profiles in kinase and safety screens. In a xenograft mouse model of AML, TH9619 significantly decreased plasma thymidine levels, reduced tumor burden and prolonged survival in a dose-dependent manner, outperforming standard of care compound AraC, with CETSA assays confirming intratumor target engagement of MTHFD2 in TH9619-treated animals.

In summary, the findings of this study describe a new role for MTHFD2 in maintaining genome stability through supporting *de novo* thymidylate synthesis for DNA replication in cancer cells. This work also presents the development of first-in-class, potent and cell active MTHFD2i with promising clinical potential. Extensive chemical and biological characterization of this new class of MTHFD2i provides compelling evidence of their antitumor efficacy with a clearly defined mechanism of action and a cancer-enriched activity profile, making these compounds attractive to improve selectivity and efficacy of current cancer therapy.

2.3.4 Paper IV: Targeting MTHFD2 impairs homologous recombination and sensitizes cancer cells to PARP inhibitors

From our lab, two previous genome-wide siRNA screens to identify novel HR repair factors independently discovered MTHFD2 among their hits (450,454). Analysis of gene array data from radioresistant patients also revealed MTHFD2 mRNA to be upregulated in response to

radiotherapy (415). Together with more recent reports of MTHFD2 supporting non-canonical nuclear functions (211,212), including our findings from **Paper III**, the evidence pointed towards a potential role for MTHFD2 in HR repair of DSBs.

To validate *in vitro* the upregulation of MTHFD2 mRNA upon IR in patients, we monitored MTHFD2 protein levels following hydroxyurea (HU)- and IR-induced DSBs in U2OS cells and found that both DSB-inducing treatments resulted in accumulation of overall cellular MTHFD2 protein levels. Upon inhibition of upstream DDR kinases ATM, ATR and DNA-PK prior to IR treatment, we observed that MTHFD2 protein accumulation was abolished in ATM- and DNA-PK-inhibited cells, but not in ATR-inhibited cells, consistent with a potential role in ATM- and DNA-PK-mediated DSB repair. Using subcellular fractionation, we were able to observe a distinct association between MTHFD2 and chromatin in response to IR, which was suppressed upon ATM or MTHFD2 inhibition. Taken together, these data supported a role for MTHFD2 in DSB repair under ATM signaling control.

Our hypothesis was that MTHFD2 supported HR repair of DSBs, which we tested by means of the DR-GFP assay. Depletion of MTHFD2 drastically reduced HR activity by over 80% in U2OS cells, to a comparable extent as loss of the critical HR factor RAD51. We observed that HR activity in these cells correlated with MTHFD2 protein levels, recognizing MTHFD2 expression as a determinant of HR proficiency in cancer cells. Next, we evaluated the effect of MTHFD2 depletion on the recruitment of HR repair factors to DSBs, namely RPA32 and RAD51. Silencing of MTHFD2 prior to IR significantly decreased the focal accumulation and nuclear intensity of both RPA32 and RAD51, implying a role for MTHFD2 in HR upstream of DNA resection.

We then evaluated the effect of MTHFD2 on the late-stage response to IR, including cell cycle arrest and clearance of DNA damage. Depletion of MTHFD2 upon IR resulted in a larger fraction of cells arrested in G2/M phase and a greater proportion of cells with unresolved DNA damage 24 h post-IR as compared to IR-only control samples. Pre-treatment with MTHFD2i also induced a dramatic increase in γH2AX levels 24 h post-IR.

Given the cancer-preferential expression pattern of MTHFD2, we sought to investigate whether targeting MTHFD2 could induce cancer-specific HR deficiency and sensitize cancer cells but not normal cells to PARP inhibitors. We assessed apoptosis levels in HR proficient THP-1 cells upon treatment with MTHFD2i and PARP inhibitor olaparib, as single agents or in combination, and found that combination of MTHFD2i and olaparib synergized to induce cell death to a much larger extent than either agent alone. Compared to non-transformed LCL cells, the synergistic cell killing upon MTHFD2i and olaparib combination was apparent only in the transformed cells, in line with our hypothesis that cancer enriched MTHFD2 expression would determine sensitivity to the combination treatment. In an animal model of AML, combination of MTHFD2i and PARP inhibitor talazoparib synergistically delayed tumor growth at concentrations that had no monotherapy effect.

In conclusion, this study presents another role for MTHFD2 in genome maintenance, this time in supporting ATM-mediated HR repair of DSBs. Moreover, we provide *in vitro* and *in vivo* evidence to propose targeting of MTHFD2 as a novel strategy to induce HR deficiency specifically in tumor cells to further sensitize them to DDR agents such as PARP inhibitors.

2.4 DISCUSSION AND FUTURE PERSPECTIVES

2.4.1 PFKFB3 inhibitors as chemo- and radiosensitizers

When the premise of this thesis was established, PFKFB3 had gained notoriety as an important glycolytic regulator in tumors and attempts to develop potent inhibitors with anticancer efficacy had already shown promising proof-of-concept results (74,103,110). Nevertheless, the effects of different inhibitors were varied and additional nuclear and non-glycolytic roles of the enzyme were just starting to be discovered (124), suggesting there was more to the picture than we had previously thought.

Paper I highlighted a previously undescribed role for PFKFB3 in supporting ATM-mediated HR repair of IR-induced DSBs by promoting local dNTP production through its interaction with RRM2. These findings were in line with known ATM regulation of metabolic rewiring and glycolytic pathways to stimulate nucleotide and antioxidant synthesis upon DDR signaling (322,455,456).

Moreover, the development of KAN757 as a potent and selective PFKFB3 inhibitor allowed us to investigate the potential of targeting PFKFB3 as a radiosensitizing strategy. As previously discussed in this thesis, effective therapeutic radiation doses are often limited in the clinic by low normal tissue tolerance (340). Additionally, metabolic alterations that promote glycolytic, hypoxic and acidotic microenvironments have recently been implicated in resistance to radio- and chemotherapy (86,93,350,351). Our results from **Paper I** showed that KAN757 could preferentially sensitize transformed cells to radiotherapy, likely through combined effects on general glycolysis (i.e., energy production, biosynthesis and ROS) and DNA repair via PFKFB3-mediated dNTP production at DSB sites. Whereas previous glycolytic inhibitors such as 2-DG have not been successful due to their systemic hypoglycemic side-effects (395), PFKFB3 inhibitors provide an elegant alternative to target glycolysis and DNA repair specifically in cancer cells where PFKFB3 is upregulated. In particular, KAN757 has recently been shown to display cancer-specific cytotoxicity on patient-derived intestinal cancer organoids, as well as a highly tolerable profile in mouse models without systemic toxicity (457), further strengthening the concept of achieving tumor selectivity through the use of PFKFB3 inhibitors.

In view of the high plasticity and adaptability of metabolic pathways in cancer cells, combination strategies that target several orthogonal tumor-promoting processes in parallel have the potential to improve sensitivity and efficacy, while limiting the emergence of

resistance. PFKFB3 inhibition has the potential to enhance the therapeutic benefit of many other anticancer strategies beyond radiotherapy (352,355,385).

Targeting glycolysis concomitantly with receptor tyrosine kinase or serine/threonine kinase signaling has been largely explored in the context of constitutive kinase activation driven cancers like chronic myeloid leukemia, melanoma and non-small cell lung cancer, as well as hormone responsive tumors like breast cancer, with promising results in clinical trials (84,97,102,103,111). Although new data suggests that the compounds used in these studies (3PO, PFK15 and PFK-158) may inhibit glycolysis via PFKFB3-independent mechanisms (398–400), similar synergies could still hold true for PFKFB3 inhibitors.

Gynecological and colorectal cancers have also been shown to benefit from PFKFB3 inhibition in combination with platinum drugs, as PFKFB3 mediates platinum resistance (86,109,458). While platinum drugs acetylate PFKFB3 to disrupt nuclear localization signal activity and mediate post-exposure resistance through its cytosolic function (86), pre-treatment with PFKFB3 inhibitors prior to platinum drug therapy could also improve therapeutic outcome by disabling HR repair as described in **Paper I**, which is a key pathway involved in the repair of deleterious platinum-induced interstrand crosslinks (ICLs) (459). Indeed, a recent report validated this hypothesis by showing that reduced PFKFB3 activity resulted in increased platinum sensitivity of endometrial cancer cells *in vitro* and *in vivo* (125). Furthermore, AMPK-mediated glycolysis switch promoting mitotic arrest escape upon microtubule poisons could be another way in which PFKFB3 inhibition may synergize with established chemotherapeutics to enhance efficacy and prevent tumor escape (93).

Moreover, additional combination strategies could be devised by exploiting the cancer's own ability to rewire its metabolism and modulate its microenvironment. For example, therapies which induce hypoxic conditions would increase cellular dependence on upregulation of glycolysis, making cancer cells particularly sensitive to PFKFB3 inhibition. Modulating the pH of the tumor microenvironment to counter lactic acidosis would also reduce the resistance to glucose deprivation induced cell death and provide a context where PFKFB3 inhibition would have maximal effect on cancer killing (460,461). Finally, through its effects on immune and angiogenic processes, PFKFB3 inhibition have also been shown to dampen tumorigenic inflammation, inhibit angiogenesis and migration, and improve antitumor activity of immune checkpoint inhibitors *in vitro*, *in vivo* and in clinical trials (75,106,462,463).

In summary, our findings from **Paper I** open the door to new and distinct research avenues for PFKFB3 in DNA replication and repair, and provide the scientific community with a new class of potent and cell active PFKFB3 inhibitors with proven utility as investigational tools and promising clinical potential, as described above.

2.4.2 MTHFD2 in nucleotide synthesis protein complexes

While the role of one-carbon metabolism in nucleotide synthesis has been widely described in the literature (128,150,464), the specific contribution of MTHFD2 has been mostly implied from correlation studies based on general rearrangements of metabolic networks. Data from our lab and others strongly support a role for MTHFD2 locally at sites of nucleotide synthesis (200,211), including our findings in **Papers III and IV**, yet direct evidence of MTHFD2 protein being found at these sites is still lacking.

De novo purine and pyrimidine synthesis are long, multi-step processes requiring the concerted action of many different metabolic enzymes to catalyze individual biochemical reactions. To maximize the efficiency of these processes, enzymes involved in nucleotide synthesis have been described to form functional multi-protein complexes (215,465). Results from **Paper III** implicate MTHFD2 in *de novo* thymidylate synthesis, which together with observations of its nuclear localization and its association to chromatin upon DNA damage observed in **Paper IV**, suggest that it may be part of the nuclear dTMP synthesis complex, similar to other folate enzymes such as SHMT1, SHTM2 and MTHFD1 (215,217,275,466). Complementarily, preliminary data from our lab indicates that overexpression of MTHFD2 is enough to stimulate purine synthesis and that MTHFD2 enzymatic activity is required for maintenance and correct assembly of purinosomes (data not shown), in line with studies demonstrating the tight crosstalk between mitochondrial one-carbon metabolism and *de novo* purine synthesis (200).

Subsequent insights from the MTHFD2i developed in **Paper III** show that their effects on cell viability can be potentiated by purine synthesis inhibitors (232), while they cannot be rescued by addition of purine intermediates 5-amino-4-imidazole-carboxamide (AICA)-riboside, adenine or adenosine (data not shown), suggesting their mechanism of action is mainly through alterations of *de novo* thymidylate synthesis. This is in line with siRNA data showing viability of MTHFD2-depeleted cells cannot be rescued by hypoxanthine. However, and in contrast to HL-60 cells, in SW620 and HCT-116 cells where thymidine rescue is partial, concomitant addition of hypoxanthine results in complete viability rescue (data not shown). This suggests that while targeting MTHFD2 can affect both purine and pyrimidine synthesis pathways, the extent to which they contribute to the MTHFD2i phenotype is likely cell and genetic context dependent.

Part of the original thesis plan was to study the potential protein-protein interactions between MTHFD2 and other members of the thymidylate and purine biosynthesis complexes in the nucleus and mitochondria using subcellular fractionation, co-immunoprecipitation and isolation of proteins on nascent DNA (iPOND) (467) techniques, as well as efforts to purify and co-crystallize these complexes together with our structural biology collaborators. However, this remains to be investigated and constitutes an interesting line of research for the continuation of the project.

2.4.3 MTHFD2 in DNA repair mechanisms beyond HR

In **Paper IV**, we described a new role for MTHFD2 in supporting HR repair of DSBs and proposed a potential application of MTHFD2 targeting to introduce HR deficiency specifically in cancer cells. We observed that MTHFD2 induction upon IR was affected by ATM inhibition, in line with the described function in HR, yet we also observed that this accumulation was abolished by inhibition of DNA-PK, an important regulator of NHEJ. Preliminary results using a DR-GFP assay variant to study DNA end-joining (EJ-GFP) revealed that siRNA depletion of MTHFD2 also significantly affected NHEJ repair potential of U2OS cells (data not shown). Taken together, this suggests an involvement of MTHFD2 prior to repair pathway choice.

Since we established in **Paper IV** that MTHFD2 supports repair of DSBs downstream of ATM and DNA-PK kinase signaling but upstream of RPA and RAD51 recruitment, we suspected it could be related to incorrect or inefficient DNA resection. This would be in line with our model involving MTHFD2 in repair pathway choice, as DNA resection is a key determinant in the commitment to HR (468). Recently, MTHFD2 was found to mediate activation of DNA resection endonuclease EXO1 via CDK1 in mouse pluripotent stem cells (469). Both DDR and one-carbon metabolism pathways are highly conserved in mammals, implying the MTHFD2-CDK1-EXO1 interaction may also be maintained in human cells, thereby explaining our observations. Moving forward with the MTHFD2 project, it will be important to (1) determine whether this mechanism translates in the human context, (2) further characterize the involvement of MTHFD2 in NHEJ repair, and (3) investigate its potential effects on alternative DNA repair pathways.

2.4.4 Potential combination strategies for MTHFD2i

To further validate the proposed mechanism of action of MTHFD2i presented in **Paper III**, we predicted and confirmed that combination of MTHFD2i and dUTPase inhibitors would potentiate deleterious misincorporation of uracil into replicating DNA and enhance cancer cell killing. Indeed, dUTPase seems to have a critical role in promoting the resistance and survival of cancer cells upon treatment with TS inhibitors such as 5-FU, 5-FUdR and raltitrexed (246,258,470). In clinical trials, dUTPase inhibitors such as TAS-114 have shown anticancer efficacy, however when combined with 5-FU result in increased levels of severe grade 3/4 toxicity (471–473). These limiting adverse effects are likely the result of TS expression across healthy tissues, which presents an opportunity for inhibitors of the cancer-enriched MTHFD2 to achieve a similar thymineless death phenotype specifically in tumor cells and sensitizing them, and not healthy cells, to dUTPase inhibition, effectively reducing severe toxicity.

Likewise, in **Paper III** we tested the potential combination of MTHFD2i and inhibitors of the replication stress response, notably ATR. CHK1 and WEE1 inhibitors, to confirm the proposed role of MTHFD2 in supporting DNA replication. We demonstrated great synergistic potential between MTHFD2i and inhibitors of the ATR signaling cascade in inducing cancer cell killing,

with the ability to significantly sensitize MTHFD2i-resistant U2OS cells to treatment. Development of targeted DDR inhibitors suppressing ATR signaling has grown exponentially in recent years, demonstrating promising antitumor activity and combination potential in Phase I and Phase II clinical trials (240,241,300,301,314). Combination of potent ATR inhibitor VE-822 with DNA damaging agent topotecan has shown great anticancer efficacy against advanced solid tumors in Phase I trials (474). Unfortunately, similar to dUTPase and 5-FU, the study also demonstrated increased levels of dose-limiting grade 3/4 hematological toxicities which followed the pattern of topotecan toxicity. Therapeutic restriction due to lack of cancer specificity by topotecan represents yet another instance where MTHFD2i could prove useful in potentiating the clinical efficacy of ATR inhibition with fewer side effects.

Paper IV described the role of MTHFD2 in DSB repair, implying potential synergies for MTHFD2i with radiotherapy and PARP inhibitors. Early *in vivo* results for the MTHFD2i and talazoparib combination, while positive, were not striking. This could be due to the choice of a weaker MTHFD2i or a suboptimal AML model. It would be interesting to test the combination in HR proficient vs. deficient cells and optimize additional experimental parameters to evaluate the full potential of this promising combination therapy. Other studies targeting MTHFD2 in combination with radiotherapy also found a radiosensitization effect when combined with ROS inducing beta-lapachone (475). As observed by the near-complete rescue of MTHFD2i cancer cell killing by thymidine supplementation in **Paper III**, we conclude that this class of inhibitors acts mainly through their effects on DNA replication in AML cells. Nevertheless, MTHFD2i combination regimens in other tumor types such as breast, ovarian, colorectal and prostate cancers upon development of resistance to PARP inhibition may be worth evaluating.

Additional studies have also explored the potential of combining MTHFD2 inhibition with secondary metabolic targets such as inhibition of SHMT1 to prevent rescue through cytosolic reversal of one-carbon directionality (181,384), and inhibition of *de novo* purine synthesis enzyme phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazole-succinocarboxamide synthase (PAICS) (232). More recent observations have also suggested roles for MTHFD2 in conferring higher ROS tolerance upon cisplatin treatment (476), and in promoting tumorigenesis via PD-L1 expression and stabilization of MYC (477). Consequently, there is promising potential for MTHFD2i as sensitizers to platinum drugs and cancer immunotherapy which deserves further validation. Finally, CRISPR-Cas9 screens to find additional synthetic lethal interactions with MTHFD2i beyond the ones described above would be of particular interest.

2.4.5 Folate transport and biomarkers to predict MTHFD2i response

Among the biggest challenges in cancer drug development is finding out which patients are most likely to benefit from a given therapy. The success of recent targeted therapies has relied mainly on selecting patients by target gene expression levels or increased activity in associated pathways. Unfortunately, the same strategy has not proven as predictive for metabolic cancer

therapies (30). For example, DHFR and TS targets are present and overexpressed in virtually all cancers, yet the efficacy of antifolates and 5-FU across tumor types varies considerably. Similarly, we have found that while MTHFD2 is the most common overexpressed metabolic target in cancer, response to MTHFD2i is far from universal.

In **Paper III**, *in vitro* pharmacological evaluation of MTHFD2i revealed the compounds had poor membrane permeability due to their highly polar hydrophilic structures, yet retained potent cell activity, suggesting compound uptake occurs via active transport similar to other folate-based metabolites and antifolate drugs.

Besides the main folate carrier, RFC, two other main folate transport systems exist in cells: folate receptors (FRs) and the proton-coupled folate transporter (PCFT) (478–480). Additionally, mitochondrial folate trafficking is mediated by the mitochondrial folate transporter (MFT) (481). The differential regulation, expression and activity levels of these various carrier systems across tissues are important determinants of folate uptake, and together with FPGS, gamma-glutamyl hydrolase (GGH) and folate hydrolase 1 (FOLH1), modulate intracellular concentrations of folate molecules (160,162,482,483).

Evaluation of our MTHFD2i and their structural features allowing or impeding polyglutamylation by FPGS revealed that the ratio between cellular and biochemical potency favored those compounds which could be polyglutamylated, in line with what is known about intracellular retention of folate derivatives. While we have confirmed TH9619 to be a substrate for FPGS, the extent to which the different folate transporters are involved in mediating TH9619 transport and efficacy remains to be determined. Structurally similar methotrexate and pemetrexed are mainly transported by RFC and PCFT (482), and so future efforts to investigate TH9619 transport mechanisms should consider these carriers first. Understanding the intracellular transport and subcellular distribution of MTHFD2i is key to isolate compartment-specific contributions to their anticancer efficacy, to determine additional response-predictive factors, and to tackle potential resistance mechanisms.

Factors involved in polyglutamylation may also predict response to MTHFD2i. FPGS and GGH, as master regulators of folate polyglutamylation and intracellular retention, are also predictors of treatment outcome upon antifolate and 5-FU therapy in gastric and lung cancer clinical trials (484,485). FOLH1, also known as prostate-specific membrane antigen (PSMA), has been used as a biomarker in prostate and breast cancer, with higher PSMA levels correlating with poor clinical outcome and increased relapse frequencies (486). These factors should also be considered in the selection of potentially MTHFD2i-sensitive tumors.

Finally, given the high level of redundancy and plasticity within the one-carbon metabolic pathways, expression levels of other one-carbon metabolism enzymes may also influence response to MTHFD2i. In particular, cytosolic MTHFD1 and SHTM1 have been shown to mediate one-carbon metabolic flux reversal upon loss of MTHFD2 (181). Empirically, HL-60 cells demonstrate the best response to our MTHFD2i among the cells tested thus far, and coincidentally also have display high levels of MTHFD2 expression together with low

MTHFD1 and SHMT1 protein levels (see **Paper III**). Constitutive KRAS activation and signaling has also been described to enhance the dependency on folate metabolism and predict response to MTHFD2i *in vitro* and *in vivo* (205,206,222).

Altogether, the evidence shows that response to metabolic target inhibition, and in this case to MTHFD2i, is extremely complex. Above we have outlined perhaps the biggest determinants of MTHFD2i efficacy besides MTHFD2 expression, however future studies are needed to uncover additional mechanisms and genetic contexts arbitrating response to MTHFD2i.

One major caveat of antifolate drug development is the lack of optimal pre-clinical models for drug evaluation. Murine models, which are the backbone of translational research, are poorly suited for demonstrating antifolate efficacy as their intrinsic levels of folate and nucleoside metabolites are orders of magnitude higher than those found in humans (487). These metabolic differences often mask any potential effects and complicate pre-clinical evaluation of antifolates. The workaround used in **Paper III** was to feed the mice low-folate chow for two weeks prior to study start and throughout the duration of the study, to reduce the animals' folate and thymidine levels enough. However, prolonged folate deficiency in the mice can also lead to a wide range of toxicities and may confound the efficacy and safety profile of the candidate drugs. In lieu of established rodent pre-clinical models, new technological approaches such as *ex vivo* drug screening may hold the key to improve pre-clinical evaluation of MTHFD2i efficacy and safety (488).

Despite a great number of recent discoveries, there is still a pressing need to improve our understanding of MTHFD enzymes, particularly in terms of designing new drugs targeting specific isozymes and associated molecular mechanisms. As for the future of MTHFD2i, we are currently working towards progressing suitable candidates into clinical development. Early *in vivo* characterization of TH9619 is promising, but further experiments are required to find alternative pre-clinical models and to optimize drug response prediction. Of immediate interest is the elucidation of MTHFD1 and MTHFD2 contributions to the anticancer effect of TH9619, which may have direct implications for response markers and selection of potentially sensitive cancers. Nevertheless, TH9619 represents a high quality MTHFD1/2 probe molecule that can continue to be used to uncover the complexities of established and non-canonical MTHFD biology in physiologically relevant contexts.

3 ACKNOWLEDGEMENTS

To my main supervisor **Thomas** – thank you for taking a chance on me and giving me the opportunity to develop as a scientist and as a professional in your group. Thanks for trusting me and allowing me the freedom to follow my interests and shape my own path.

To my co-supervisors and team leaders **Oliver** and **Nina**, thank you for the hands-on support and guidance all these years. Special thanks to **Oliver** for helping me to say "no" and pushing me through the finish line.

To **Ulrika**, thank you for stepping in as honorary supervisor and always keeping your door open. I have learned a great deal on people and crisis management from you. My best wishes for your future adventures.

To **Kristina**, **Sabina**, **Flor**, **Mari**, **Lollo**, my sincere gratitude and admiration for your tireless work behind the scenes. None of this would be possible without your infinite knowledge and patience. Thank you!

To my lab squad, my mates in the trenches: **Aleksandra**, **Anna**, **Jemina**, and **Stella** – what can I say besides: I owe you my life! Thanks for always being there to support, encourage, motivate, and focus me. In between jokes, chicken videos, panic attacks, late nights and weekends in the lab, angry emails, burgers and beers, and a million other memories, a real bond of friendship was formed based on the genuine desire to see each other succeed. I am so grateful to have shared this once-in-a-lifetime rollercoaster experience with you. I don't have to tell you, you already know.

To all the PhD fellows I had the chance to share the lab with, **Petra**, **Bishoy**, **Marianna**, **Johan**, **Linda**, **Nick**, **Saeed** and **Anna** – thank you for showing me the ropes and always supporting me when I asked for help. I learned so much from all of you, on and beyond the bench. I wish you nothing but success and opportunities to grow in the future.

To the **Helleday lab** members and our collaborators, past and present; thank you all for being part of my journey. Special mention to my onboarding committee: **Sofia Henriksson**, **Petra Groth** and **José Calderón**– thank you for making me feel welcome in the lab and having the patience to guide a lost sheep through the jungle. Your support and encouragement will never be forgotten. To my project colleagues, especially **Evert**, **Elisée**, **Ingrid**, **Therese** and **Azita** – it was a pleasure learning from and working alongside such professional and kindhearted teammates. To **Carlos**, **Sanaz** and **Miriam** – thank you for sharing your joy, your generosity and positivity, you were always the warm rays of sun that brightened my days.

To my friends, or the family I have found along the way. Starting with **Jessica**, pimpolla, gracias por tu amistad de décadas, por mantenerme cuerda a pesar de la distancia. Aun cuando se nos olvidaba hablar por meses, tu apoyo y nuestras atorrancias siempre hacían que el tiempo se pasara volando. To **Misty**, **Annisa**, **Devy**, **Alex**, **Arne**, **Berty** and **Magnus**, my second

family, thank you for taking me in as one of your own, for sharing in the good, the bad and the ugly. Thanks for all the dancing, the housewarming parties, the Thanksgiving parties, the Christmas parties (basically all the parties), the brunching, the noodle nights, the day drinking, the late-night drives, the trips, the memes and the board games – you guys have a special place in my heart. To **Cynthia**, **Judith** and **Flor** (yes, you get a mention twice). Yet another reason to be thankful for my time in the lab – meeting you, lovely souls. Thank you for the lunches, the trips, the hikes, the adventures, and for always having my back. You know better than most what this journey has meant to me, and I will never be able to thank you enough for sharing your friendship and selfless support with me. To my UNIL ladies **Koki**, **Stef**, **Valérie** – merci d'avoir ouvert vos cœurs à cette drôle étrangère maniaque qui connaît tous les mots croisés, qui met des bigoudis en pleine session d'examens et qui nettoie sa gomme d'effacer. Merci pour votre patience, pour les rires, pour les heures d'étude à la Banane, pour les crêpe-parties, les pique-niques et les BBQs au bord du lac. Vous êtes là depuis le début de ce voyage et j'ai hâte d'en partager bien d'autres avec vous, peu importe où dans le monde. To **Suzanna** and **Olga**, my partners in crime. I am so grateful to have found such kindred souls along my non-PhD ventures. Your support has ranged from psychotherapist to career coach and entrepreneurial co-founder, passing through every other shade of friendship in between. Thank you for your unconditional support, your objective and honest advice, and your pep talks, you made this journey feel much less lonely and much more fun.

To my **180 Degrees Consulting** family – thank you for giving me one of the greatest opportunities to learn from and share with so many talented and exceptional people. Thank you for bringing new friends and new perspectives into my life, you guys have been instrumental to my personal and professional development, and have contributed to this thesis more than you know.

To my mentor **Milica**, and my unofficial advisers **Claudia**, **Beata**, **Catarina**, **Irina** and the rest of the **PWN Stockholm** community – thank you for being such strong, driven and unapologetic role models, for supporting me and believing in me even when I doubted myself, and for showing me that in the world of success and professionalism there is always room for empathy and kindness. You are inspiring women.

To the **Karolinska Institutet Career Service**, my wonderful colleagues **Kerstin**, **Victoria**, **Anethe** and **Ayla**, and the myriad of lecturers, coaches, alumni, and speakers I have had the pleasure of listening to during my time at KI. You have been an infinite source of inspiration and renewed motivation, always reminding me why I chose to do a PhD in the first place and giving me the tools to get there.

To the unsung heroes fighting for the mental health and wellbeing of PhD students, those who pick up the pieces following years of undue stress, burnouts, insomnia, depression, anxiety, and all their associated stigmas, too common among doctoral students and academia at large. **Åsa Samuelsson**, **Emmy Lindedal**, and **Patrik Emanuelsson** – thank you for putting the pieces back together. I wish your roles did not have to exist, but I am grateful they did. I would have never made it without you.

To the mentors, educators and supervisors who stoked and nurtured my enthusiasm for STEM throughout the years: **Viorica Triculescu**, **Olga Mow**, **Michael Meyer** and **Luigi Maddaluno** – thank you for your patience, your resilience, your wisdom and above all your willingness to share your passion and scientific discipline with me.

A ma famille d'accueil, **Nea Nicki**, **Mihaela**, **Emma**, **Vincent** et **Raphaël**. Vous êtes simplement les meilleurs. Je serais toujours reconnaissante pour votre aide, votre soutien, vos encouragements. Votre confiance en moi et en ce que je pouvais accomplir a toujours renouvelé mes forces. Chaque seconde partagée avec vous m'a rappelé vers quoi je travaillais, et maintenant que je suis à la ligne d'arrivée, je ne trouve plus les mots pour remercier votre amour et votre patience. Merci.

A las **familias Villarreal**, **Bonagas** y **Aizpurúa** de Caldera y Horconcitos, Provincia de Chiriquí, Panamá. Gracias infinitas por su sacrificio y entrega a lo largo de las tantas generaciones que hicieron posible que llegara hasta aquí. Si hoy cumplo esta meta es porque ustedes me han traído a tres pasos de la cima, y me llena el alma de alegría poder compartir el orgullo de este logro con todos ustedes.

A mi padre, **Javier**, gracias por heredarme tu sabiduría, ingenio, pasión y esmero por tu profesión, por cultivar mi intelecto y pensamiento crítico, por transmitir la visión de mis abuelos de invertir siempre en la educación, y por siempre apoyar mis ambiciones y decisiones, aunque éstas me llevaran lejos de nuestra tierra. He aquí uno de los más grandes frutos de todas esas inversiones, ojalá algún día sea de beneficio para aquellos que más lo necesiten.

A mis tías, madrinas y segundas madres, **Marlene** y **Margarita**. Gracias por criarme como una hija más, por brindarme todo su amor y apoyo, y acompañarme en todas las etapas de mi vida, buenas y no tan buenas. Gracias por inculcarme valores de honradez, trabajo duro, esfuerzo y abnegación que me han acompañado siempre y han hecho posible que hoy llegue a esta meta. A mi ángel de la guarda, la matriarca de la familia, la única e incomparable **Blanca Rosa** – gracias a la disciplina y la humildad que nos inculcaste es que hemos llegado mucho más lejos de lo que nadie esperaba.

A la mejor madre del mundo, **Anayansy**. Mi alcahueta, mi compinche, mi porrista estrella, mi fan número uno. Tú sabes que no me alcanzan las palabras para agradecerte todo lo que has hecho y sacrificado por mí, desde los batidos de berro hasta nuestra vajilla de plástico reciclado. Gracias por estar siempre pendiente de todo, por compartir las tristezas y las felicidades, por apoyarme y creer en mí, por darme raíces y alas. "Vendrán tiempos mejores" siempre me dijiste – pues ya llegamos!

To my sister, **Keyra**, my mini-me accomplice. Thank you for all the support and encouragement you have given me through this long and winding road. Despite the time spent apart, I hope to have been a good role model in perseverance and determination, and that you take these shared life lessons with you as you find your own path. I cannot wait to help you reach your achievements as you have helped me reach mine.

To **Alex**, my love, my rock, my funny guy – thank you for being in my corner always, for believing in me even when I lost faith, for wiping the sweat and tears out of my eyes and pushing me back in the ring a million times because you knew I could win the fight. We have come a long way; 13 years and at least a thousand 2000 km-round trips, and while this chapter could not and would not have been completed without you, I can only feel excitement for the many chapters that are still to come.

And to everyone else who in one way or another helped, enabled, provided and contributed to this work. My sincere gratitude.

"Podrán cortar todas las flores, pero no podrán detener la primavera"

– Pablo Neruda

Premio Nobel de Literatura en 1971, uno de los más grandes poetas del siglo XX, y una vida más eclipsada por el cáncer

4 REFERENCES

1. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol. 1927;8(6):519–30.
2. Koppenol WH, Bounds PL, Dang C V. Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer. 2011;11(5):325–37.
3. Warburg O. On the origin of cancer cells. Science (80-). 1956;123(3191):309–14.
4. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61(5):759–67.
5. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. 1983;304(18):596–602.
6. Varmus H, Pao W, Politi K, Podsypanina K, Du YCN. Oncogenes come of age. Cold Spring Harb Symp Quant Biol. 2005;70:1–9.
7. Ward PS, Thompson CB. Metabolic Reprogramming: A Cancer Hallmark Even Warburg Did Not Anticipate. Cancer Cell. 2012;21(3):297–308.
8. Liberti M V., Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016;41(3):211–8.
9. Jiang BH, Agani F, Passaniti A, Semenza GL. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: Involvement of HIF-1 in tumor progression. Cancer Res. 1997;57(23):5328–35.
10. King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: Linking mitochondrial dysfunction and cancer. Oncogene. 2006;25(34):4675–82.
11. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci. 2010;107(5):2037–42.
12. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev. 2010;20(1):51–6.
13. Robertson-Tessi M, Gillies RJ, Gatenby RA, Anderson ARA. Impact of metabolic heterogeneity on tumor growth, invasion, and treatment outcomes. Cancer Res. 2015;75(8):1567–79.
14. Yu L, Lu M, Jia D, Ma J, Ben-Jacob E, Levine H, et al. Modeling the genetic regulation of cancer metabolism: Interplay between glycolysis and oxidative phosphorylation. Cancer Res. 2017;77(7):1564–74.
15. Shim H, Dolde C, Lewis BC, Wu C-S, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: Implications for tumor metabolism and growth. Natl Institutes Heal. 1997;94(June):6658–63.
16. Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem. 2000;275(29):21797–800.
17. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. Semin Cancer Biol. 2005;15(4):300–8.
18. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An Integrated Genomic Analysis of Human GBM. Science (80-). 2008;321(5897):1807–12.
19. Gross S, Cairns RA, Minden MD, Driggers EM, Bittinger MA, Jang HG, et al. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. J Exp Med. 2010;207(2):339–44.
20. Bayley JP, Devilee P. Warburg tumours and the mechanisms of mitochondrial tumour suppressor genes. Barking up the right tree? Curr Opin Genet Dev. 2010;20(3):324–9.
21. Jeon S-M, Hay N. Expanding the concepts of cancer metabolism. Exp Mol Med. 2018;50(4):10–2.
22. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
23. DeBerardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. Curr Opin Genet Dev. 2008;18(1):54–61.
24. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell. 2008;134(5):703–7.
25. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg Effect: the metabolic requirements of cell proliferation. Science (80-). 2009;324(5930):1029–33.
26. Levine AJ, Puzio-Kuter AM. The Control of the Metabolic Switch in Cancers by Oncogenes and Tumor. Science (80-). 2010;330(December):1340–4.
27. Vander Heiden MG, DeBerardinis RJ. Understanding the Intersections between Metabolism and Cancer Biology. Cell. 2017;168(4):657–69.
28. Livshits Z, Rao RB, Smith SW. An approach to chemotherapy-associated toxicity. Emerg Med Clin North Am. 2014;32(1):167–203.
29. Martinez-Outschoorn UE, Peiris-Pagés M, Pestell RG, Sotgia F, Lisanti MP. Cancer metabolism: A therapeutic perspective. Nat Rev Clin Oncol. 2017;14(1):11–31.
30. Luengo A, Gui DY, Vander Heiden MG. Targeting metabolism for cancer therapy. Cell Chem Biol. 2017;24(9):1161.
31. Mullard A. FDA approves first-in-class cancer metabolism drug. Nat Rev Drug Discov. 2017;16(9):593–593.
32. Mullard A. Cancer metabolism pipeline breaks new ground. Nat Rev Drug Discov. 2016;15(11):735–7.
33. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Sci Adv. 2016;2(5):e1600200.
34. Thompson CB. Metabolic Enzymes as Oncogenes or Tumor Suppressors. N Engl J Med. 2009;360(8):813–5.
35. Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T, et al. Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. J Biol Chem. 2006;281(24):16768.
36. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T. Quantitative Metabolome Analysis Using Capillary Electrophoresis Mass Spectrometry. J Proteome Res. 2003;2(5):488–94.
37. Choi C, Ganji SK, DeBerardinis RJ, Hatanpaa KJ, Rakheja D, Kovacs Z, et al. 2-Hydroxyglutarate detection by magnetic resonance spectroscopy in IDH-mutated patients with gliomas. Nat Med. 2012;18(4):624–9.
38. Tomita M, Kami K. Systems Biology, Metabolomics, and Cancer Metabolism. Science (80-). 2012;336(May):990.
39. Yang M, Soga T, Pollard PJ. Oncometabolites: Linking altered metabolism with cancer. J Clin Invest. 2013;123(9):3652–8.

40. Mullen AR, DeBerardinis RJ. Genetically-defined metabolic reprogramming in cancer. Trends Endocrinol Metab. 2012;23(11):552–9.
41. Pollard PJ, Brière JJ, Alam NA, Barwell J, Barclay E, Wortham NC, et al. Accumulation of Krebs cycle intermediates and over-expression of HIF1α in tumours which result from germline FH and SDH mutations. Hum Mol Genet. 2005;14(15):2231–9.
42. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, et al. The Common Feature of Leukemia-Associated IDH1 and IDH2 Mutations Is a Neomorphic Enzyme Activity Converting α-Ketoglutarate to 2-Hydroxyglutarate. Cancer Cell. 2010;17(3):225–34.
43. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009;462(7274):739–44.
44. Ishii N, Tsuda M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, et al. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature. 1998;394(August):694–7.
45. Ishii T, Yasuda K, Akatsuka A, Hino O, Hartman PS, Ishii N. A mutation in the SDHC gene of complex II increases oxidative stress, resulting in apoptosis and tumorigenesis. Cancer Res. 2005;65(1):203–9.
46. Roboz GJ, DiNardo CD, Stein EM, de Botton S, Mims AS, Prince GT, et al. Ivosidenib induces deep durable remissions in patients with newly diagnosed IDH1-mutant acute myeloid leukemia. Blood. 2020;135(7):463–71.
47. Harris AL. Development of cancer metabolism as a therapeutic target: new pathways, patient studies, stratification and combination therapy. Br J Cancer. 2020;122(1).
48. Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza AL, et al. Metabolite Profiling Identifies a Key Role for Glycine in Rapid Cancer Cell Proliferation. Science (80-). 2012;336(May):1040–4.
49. Vazquez A, Kamphorst JJ, Markert EK, Schug ZT, Tardito S, Gottlieb E. Cancer metabolism at a glance. J Cell Sci. 2016;129(18):3367–73.
50. Cuezva JM, Krajewska M, De Heredia ML, Krajewski S, Santamaría G, Kim H, et al. The bioenergetic signature of cancer: A marker of tumor progression. Cancer Res. 2002;62(22):6674–81.
51. Guppy M, Greiner E, Brand K. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. Eur J Biochem. 1993;212(1):95–9.
52. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. Cell Metab. 2008;7(1):11–20.
53. Pfeiffer T, Schuster S, Bonhoeffer S. Cooperation and competition in the evolution of ATP-producing pathways. Science (80-). 2001;293(5534):1436.
54. Weber G. Enzymology of cancer cells. N Engl J Med. 1977;296(9):486–93.
55. Van Schaftingen E, Jett MF, Jue L, Hers HG. Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. Proc Natl Acad Sci U S A. 1981;78(6 I):3483–6.
56. Pilkis SJ, Claus TH, Kurland IJ, Lange AJ. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: A metabolic signaling enzyme. Annu Rev Biochem. 1995;64:799–835.
57. Okar DA, Lange AJ. Fructose-2,6-bisphosphate and control of carbohydrate metabolism in eukaryotes. BioFactors. 1999;10(1):1–14.
58. El-Maghrabi MR, Noto F, Wu N, Manes N. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: suiting structure to need, in a family of tissue-specific enzymes. Curr Opin Clin Nutr Metab Care. 2001;4:411–8.
59. Okar DA, Lange AJ, Manzano À, Navarro-Sabatè A, Riera L, Bartrons R. PFK-2/FBPase-2: Maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci. 2001;26(1):30–5.
60. Sakakibara R, Kato M, Okamura N, Nakagawa T, Komada Y, Tominaga N, et al. Characterization of a human placental fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase. J Biochem. 1997;122(1):122–8.
61. Minchenko O, Opentanova I, Caro J. Hypoxic regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1-4) expression in vivo. FEBS Lett. 2003;554(3):264–70.
62. Minchenko O, Opentanova I, Minchenko D, Ogura T, Esumi H. Hypoxia induces transcription of 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase-4 gene via hypoxia-inducible factor-1α activation. FEBS Lett. 2004;576(1–2):14.
63. Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, et al. Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene: Its possible role in the warburg effect. J Biol Chem. 2002;277(8):6183–7.
64. Fukasawa M, Tsuchiya T, Takayama E, Shinomiya N, Uyeda K, Sakakibara R, et al. Identification and characterization of the hypoxia-responsive element of the human placental 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene. J Biochem. 2004;136(3):273–7.
65. Obach M, Navarro-Sabaté À, Caro J, Kong X, Duran J, Gómez M, et al. 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J Biol Chem. 2004;279(51):53562–70.
66. Bartrons R, Caro J. Hypoxia, glucose metabolism and the Warburg's effect. J Bioenerg Biomembr. 2007;39(3):223.
67. Chesney J, Clark J, Klarer AC, Imbert-Fernandez Y, Lane AN, Telang S. Fructose-2,6-bisphosphate synthesis by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) is required for the glycolytic response to hypoxia and tumor growth. Oncotarget. 2014;5(16):6670–86.
68. Wang Y, Qu C, Liu T, Wang C. PFKFB3 inhibitors as potential anticancer agents: mechanisms of action, current developments, and structure-activity relationships. Eur J Med Chem. 2020;203:112612.
69. Tauler A, Kai Lin, Pilkis SJ. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Use of site-directed mutagenesis to evaluate the roles of His-258 and His-392 in catalysis. J Biol Chem. 1990;265(26):15617–22.
70. Li L, Lin K, Pilkis J, Correia JJ, Pilkis SJ. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. The role of surface loop basic residues in substrate binding to the fructose-2,6- bisphosphatase domain. J Biol Chem. 1992;267(30):21588–94.
71. Lin K, Li L, Correia JJ, Pilkis SJ. Glu327 is part of a catalytic triad in rat liver fructose-2,6- bisphosphatase. J Biol Chem. 1992;267(10):6556–62.
72. Cavalier MC, Kim SG, Neau D, Lee YH. Molecular basis of the fructose-2,6-bisphosphatase reaction of PFKFB3:

Transition state and the C-terminal function. Proteins Struct Funct Bioinforma. 2012;80(4):1143–53.
73. Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Ther. 2008;7(1):110–20.
74. Zhu W, Ye L, Zhang J, Yu P, Wang H, Ye Z, et al. PFK15, a small molecule inhibitor of PFKFB3, induces cell cycle arrest, apoptosis and inhibits invasion in gastric cancer. PLoS One. 2016;11(9):1–14.
75. Chesney JA, Telang S, Yaddanapudi K, Grewal JS. Targeting 6-phosphofructo-2-kinase (PFKFB3) as an immunotherapeutic strategy. J Clin Oncol. 2016;34(15 Supplement):e14548.
76. Telang S, Yaddanapudi K, Grewal J, Redman R, Fu S, Pohlmann P, et al. PFK-158 is a first-in-human inhibitor of PFKFB3 that selectively suppresses glucose metabolism of cancer cells and inhibits the immunosuppressive Th17 cells and MDSCs in advanced cancer patients. Cancer Res. 2016;76(24 Supplement):B90.
77. Atsumi T, Chesney J, Metz C, Leng L, Donnelly S, Makita Z, et al. High expression of Inducible 6-phosphofructo-2-kinase / fructose-2 , 6-bisphosphatase (iPFK-2; PFKFB3) in human cancers. Cancer Res. 2002;62:5881–7.
78. Yi M, Ban Y, Tan Y, Xiong W, Li G, Xiang B. 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and 4: A pair of valves for fine-tuning of glucose metabolism in human cancer. Mol Metab. 2019;20(December 2018):1–13.
79. Minchenko OH, Ochiai A, Opentanova IL, Ogura T, Minchenko DO, Caro J, et al. Overexpression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 in the human breast and colon malignant tumors. Biochimie. 2005;87(11):1005–10.
80. Bando H, Atsumi T, Nishio T, Niwa H, Mishima S, Shimizu C, et al. Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer. Clin Cancer Res. 2005;11(16):5784–92.
81. Shi WK, Zhu XD, Wang CH, Zhang YY, Cai H, Li XL, et al. PFKFB3 blockade inhibits hepatocellular carcinoma growth by impairing DNA repair through AKT article. Cell Death Dis. 2018;9(4).
82. Kessler R, Bleichert F, Warnke JP, Eschrich K. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) is up-regulated in high-grade astrocytomas. J Neurooncol. 2008;86(3):257–64.
83. Novellasdemunt L, Obach M, Millán-Ariño L, Manzano A, Ventura F, Rosa JL, et al. Progestins activate 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) in breast cancer cells. Biochem J. 2012;442(2):345.
84. Imbert-Fernandez Y, Clem BF, O'Neal J, Kerr DA, Spaulding R, Lanceta L, et al. Estradiol stimulates glucose metabolism via 6-phosphofructo-2-kinase (PFKFB3). J Biol Chem. 2014;289(13):9440–8.
85. Marsin AS, Bouzin C, Bertrand L, Hue L. The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. J Biol Chem. 2002;277(34):30778–83.
86. Li FL, Liu JP, Bao RX, Yan G, Feng X, Xu YP, et al. Acetylation accumulates PFKFB3 in cytoplasm to promote glycolysis and protects cells from cisplatin-induced apoptosis. Nat Commun. 2018;9(1).
87. Seo M, Lee YH. PFKFB3 regulates oxidative stress homeostasis via its S-glutathionylation in cancer. J Mol Biol. 2014;426(4):830–42.
88. Herrero-Mendez A, Almeida A, Fernández E, Maestre C, Moncada S, Bolaños JP. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. Nat Cell Biol. 2009;11(6):747–52.
89. Yamamoto T, Takano N, Ishiwata K, Ohmura M, Nagahata Y, Matsuura T, et al. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nat Commun. 2014;5.
90. Yalcin A, Clem BF, Simmons A, Lane A, Nelson K, Clem AL, et al. Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. J Biol Chem. 2009;284(36):24223–32.
91. Kole HK, Resnick RJ, Van Doren M, Racker E. Regulation of 6-phosphofructo-1-kinase activity in ras-transformed rat-1 fibroblasts. Arch Biochem Biophys. 1991;286(2):586–90.
92. Telang S, Yalcin A, Clem AL, Bucala R, Lane AN, Eaton JW, et al. Ras transformation requires metabolic control by 6-phosphofructo-2-kinase. Oncogene. 2006;25(55):7225–34.
93. Doménech E, Maestre C, Esteban-Martínez L, Partida D, Pascual R, Fernández-Miranda G, et al. AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. Nat Cell Biol. 2015;17(10):1304–16.
94. Blum R, Jacob-Hirsch J, Amariglio N, Rechavi G, Kloog Y. Ras inhibition in glioblastoma down-regulates hypoxia-inducible factor-1α, causing glycolysis shutdown and cell death. Cancer Res. 2005;65(3):999–1006.
95. O'Neal J, Clem A, Reynolds L, Dougherty S, Imbert-Fernandez Y, Telang S, et al. Inhibition of 6-phosphofructo-2-kinase (PFKFB3) suppresses glucose metabolism and the growth of HER2+ breast cancer. Breast Cancer Res Treat. 2016;160(1):29–40.
96. Kumar P, Sharoyko V V., Spégel P, Gullberg U, Mulder H, Olsson I, et al. The Transcriptional Co-Repressor Myeloid Translocation Gene 16 Inhibits Glycolysis and Stimulates Mitochondrial Respiration. PLoS One. 2013;8(7).
97. Zhu Y, Lu L, Qiao C, Shan Y, Li H, Qian S, et al. Targeting PFKFB3 sensitizes chronic myelogenous leukemia cells to tyrosine kinase inhibitor. Oncogene. 2018;37(21):2837–49.
98. Zawacka-Pankau J, Grinkevich V V., Hünten S, Nikulenkov F, Gluch A, Li H, et al. Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: Targeting Warburg effect to fight cancer. J Biol Chem. 2011;286(48):41600–15.
99. Cordero-Espinoza L, Hagen T. Increased concentrations of fructose 2,6-bisphosphate contribute to the Warburg effect in phosphatase and tensin homolog (PTEN)-deficient cells. J Biol Chem. 2013;288(50):36020–8.
100. Ge X, Lyu P, Cao Z, Li J, Guo G, Xia W, et al. Overexpression of miR-206 suppresses glycolysis, proliferation and migration in breast cancer cells via PFKFB3 targeting. Biochem Biophys Res Commun. 2015;463(4):1115–21.
101. Peng F, Li Q, Sun JY, Luo Y, Chen M, Bao Y. PFKFB3 is involved in breast cancer proliferation, migration, invasion and angiogenesis. Int J Oncol. 2018;52(3):945–54.
102. Warrier G, Lanceta L, Imbert-Fernandez Y, Chesney JA. Inhibition of glucose metabolism through treatment of BRAF mutated metastatic melanoma with vemurafenib. J Clin Oncol. 2019;37(15 Supplement):e21005.
103. Telang S, O'Neal J, Tapolsky G, Clem B, Kerr A, Imbert-Ferndandez Y, et al. Discovery of a PFKFB3 inhibitor for phase I trial testing that synergizes with the B-Raf inhibitor vemurafenib. Cancer Metab. 2014;2(Suppl 1):P14.
104. Mendoza EE, Pocceschi MG, Kong X, Leeper DB, Caro J, Limesand KH, et al. Control of glycolytic flux by AMP-

activated protein kinase in tumor cells adapted to low ph1. Transl Oncol. 2012;5(3):208–16.
105. Bobarykina AY, Minchenko DO, Opentanova IL, Moenner M, Caro J, Esumi H, et al. Hypoxic regulation of PFKFB-3 and PFKFB-4 gene expression in gastric and pancreatic cancer cell lines and expression of PFKFB genes in gastric cancers. Acta Biochim Pol. 2006;53(4):789–99.
106. Han J, Meng Q, Xi Q, Zhang Y, Zhuang Q, Han Y, et al. Interleukin-6 stimulates aerobic glycolysis by regulating PFKFB3 at early stage of colorectal cancer. Int J Oncol. 2016;48(1):215–24.
107. Klarer AC, O'Neal J, Imbert-Fernandez Y, Clem A, Ellis SR, Clark J, et al. Inhibition of 6-phosphofructo-2-kinase (PFKFB3) induces autophagy as a survival mechanism. Cancer Metab. 2014;2(1):1–14.
108. Yalcin A, Solakoglu TH, Ozcan SC, Guzel S, Peker S, Celikler S, et al. 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase-3 is required for transforming growth factor β1-enhanced invasion of Panc1 cells in vitro. Biochem Biophys Res Commun. 2017;484(3):687–93.
109. Mondal S, Roy D, Sarkar Bhattacharya S, Jin L, Jung D, Zhang S, et al. Therapeutic targeting of PFKFB3 with a novel glycolytic inhibitor PFK158 promotes lipophagy and chemosensitivity in gynecologic cancers. Int J Cancer. 2019;144(1):178–89.
110. Clem BF, O'Neal J, Tapolsky G, Clem AL, Imbert-Fernandez Y, Kerr DA, et al. Targeting 6-phosphofructo-2-kinase (PFKFB3) as a therapeutic strategy against cancer. Mol Cancer Ther. 2013;12(8):1461–70.
111. Lypova N, Telang S, Chesney J, Imbert-Fernandez Y. Increased 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 activity in response to EGFR signaling contributes to non-small cell lung cancer cell survival. J Biol Chem. 2019;294(27):10530–43.
112. Hu KY, Wang DG, Liu PF, Cao YW, Wang YH, Yang XC, et al. Targeting of MCT1 and PFKFB3 influences cell proliferation and apoptosis in bladder cancer by altering the tumor microenvironment. Oncol Rep. 2016;36(2):945.
113. Fleischer M, Kessler R, Klammer A, Warnke J-P, Eschrich K. LOH on 10p14-p15 targets the PFKFB3 gene locus in human glioblastomas. Genes Chromosomes Cancer. 2011;50:1010–20.
114. Zscharnack K, Kessler R, Bleichert F, Warnke JP, Eschrich K. The PFKFB3 splice variant UBI2K4 is downregulated in high-grade astrocytomas and impedes the growth of U87 glioblastoma cells. Neuropathol Appl Neurobiol. 2009;35(6):566–78.
115. Li HM, Yang JG, Liu ZJ, Wang WM, Yu ZL, Ren JG, et al. Blockage of glycolysis by targeting PFKFB3 suppresses tumor growth and metastasis in head and neck squamous cell carcinoma. J Exp Clin Cancer Res. 2017;36(1):1–12.
116. Lu Q, Yan S, Sun H, Wang W, Li Y, Yang X, et al. Akt inhibition attenuates rasfonin-induced autophagy and apoptosis through the glycolytic pathway in renal cancer cells. Cell Death Dis. 2015;6:1–11.
117. Du JY, Wang LF, Wang Q, Yu LD. miR-26b inhibits proliferation, migration, invasion and apoptosis induction via the downregulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 driven glycolysis in osteosarcoma cells. Oncol Rep. 2015;33(4):1890–8.
118. Zheng WD, Zhou FL, Lin N. MicroRNA-26b inhibits osteosarcoma cell migration and invasion by down-regulating PFKFB3 expression. Genet Mol Res. 2015;14(4):16872–9.
119. Feng Y, Wu L. mTOR up-regulation of PFKFB3 is essential for acute myeloid leukemia cell survival. Biochem Biophys Res Commun. 2017;483(2):897–903.
120. Liu J, Liu ZX, Wu QN, Lu YX, Wong CW, Miao L, et al. Long noncoding RNA AGPG regulates PFKFB3-mediated tumor glycolytic reprogramming. Nat Commun. 2020;11(1).
121. Yalcin A, Clem BF, Imbert-Fernandez Y, Ozcan SC, Peker S, O'Neal J, et al. 6-Phosphofructo-2-kinase (PFKFB3) promotes cell cycle progression and suppresses apoptosis via Cdk1-mediated phosphorylation of p27. Cell Death Dis. 2014;5(7):1–10.
122. Wang X, Gorospe M, Huang Y, Holbrook NJ. p27(Kip1) overexpression causes apoptotic death of mammalian cells. Oncogene. 1997;15(24):2991–7.
123. Jia W, Zhao X, Zhao L, Yan H, Li J, Yang H, et al. Non-canonical roles of PFKFB3 in regulation of cell cycle through binding to CDK4. Oncogene. 2018;37(13):1685–98.
124. Franklin DA, He Y, Leslie PL, Tikunov AP, Fenger N, MacDonald JM, et al. p53 coordinates DNA repair with nucleotide synthesis by suppressing PFKFB3 expression and promoting the pentose phosphate pathway. Sci Rep. 2016;6(November):1–13.
125. Xiao Y, Jin L, Deng C, Guan Y, Kalogera E, Ray U, et al. Inhibition of PFKFB3 induces cell death and synergistically enhances chemosensitivity in endometrial cancer. Oncogene. 2021;40(8):1409–24.
126. Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature. 2011;476(7360):346–50.
127. Tedeschi PM, Markert EK, Gounder M, Lin H, Dvorzhinski D, Dolfi SC, et al. Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. Cell Death Dis. 2013;4(10):e877.
128. Locasale JW. Serine, glycine and one-carbon units: Cancer metabolism in full circle. Nat Rev Cancer. 2013;13(8):572–83.
129. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. N Engl J Med. 1948;238(23):787–93.
130. Farber S. Some observations on the effect of folic acid antagonists on acute leukemia and other forms of incurable cancer. Blood. 1949;4:160–7.
131. WHO. World Health Organization Model List of Essential Medicines. Vol. 21. 2019.
132. Bertino JR, Levitt M, McCullough JL, Chabner B. New Approaches To Chemotherapy With Folate Antagonists: Use of Leucovorin "Rescue" and Enzymic Folate Depletion. Ann N Y Acad Sci. 1971;186(1):486–95.
133. Allegra CJ, Fine RL, Drake JC, Chabner BA. The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. Evidence for direct inhibition of purine synthesis. J Biol Chem. 1986;261(14):6478–85.
134. Baram J, Allegra CJ, Fine RL, Chabner BA. Effect of methotrexate on intracellular folate pools in purified myeloid precursor cells from normal human bone marrow. J Clin Invest. 1987;79(3):692–7.
135. Walling J. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. Invest New Drugs. 2006;24(1):37–77.

136. Chattopadhyay S, Moran RG, Goldman ID. Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications. Mol Cancer Ther. 2007;6(2):404–17.
137. Elion GB, Singer S, Hitchings GH. Antagonists of nucleic acid derivatives. J Biol Chem. 1954;208(2):477–88.
138. Cheson BD. New antimetabolites in the treatment of human malignancies. Semin Oncol. 1992 Dec;19(6):695–706.
139. Johnston PG, Takimoto CH, Grem JL, Fidias P, Grossbard ML, Chabner BA, et al. Antimetabolites. Cancer Chemother Biol Response Modif. 1997;17:1–39.
140. Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D, Griesbach L, Duschinsky R, et al. Fluorinated Pyrimidines, A New Class of Tumour-Inhibitory Compounds. Nature. 1957;179(4561):663–6.
141. Elion GB. The purine path to chemotherapy. Science (80-). 1989;244(4900):41–7.
142. Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: A systematic review and meta-analysis based on aggregate data. J Clin Oncol. 2006;24(18):2903–9.
143. Parker WB. Enzymology of Purine and Pyrimidine Antimetabolites Used in the Treatment of Cancer. Chem Rev. 2009;109(7):2880–93.
144. Locasale JW, Cantley LC. Genetic selection for enhanced serine metabolism in cancer development. Cell Cycle. 2011;10(22):3812–3.
145. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer. 2011;11(2):85–95.
146. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. Nature. 2012;491(7424):364–73.
147. Shane B, Stokstad ELR. Vitamin B12-Folate Interrelationships. Annu Rev Nutr. 1985;5(1):115–41.
148. Stipanuk MH. Sulfur Amino Acid Metabolism: Pathways for Production and Removal of Homocysteine and Cysteine. Annu Rev Nutr. 2004;24(1):539–77.
149. Stover PJ. Physiology of Folate and Vitamin B12 in Health and Disease. Nutr Rev. 2004;62(6):3–12.
150. Tibbetts AS, Appling DR. Compartmentalization of Mammalian Folate-Mediated One-Carbon Metabolism. Annu Rev Nutr. 2010;30(1):57–81.
151. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem. 1990;1(5):228–37.
152. Yun J, Johnson JL, Hanigan CL, Locasale JW. Interactions between epigenetics and metabolism in cancers. Front Oncol. 2012;2(November):1–10.
153. Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 2012;23(8):853–9.
154. Katada S, Imhof A, Sassone-Corsi P. Connecting threads: Epigenetics and metabolism. Cell. 2012;148(1–2):24–8.
155. Gluckman PD. Epigenetics, the life-course and metabolic disease. Nat Rev Endocrinol. 2012;8(2):74–6.
156. Zatz M, Dudley PA, Klooga Y, Markey SP. Nonpolar Lipid Methylation. Biosynthesis of fatty acid methyl esters by rat lung membranes using S-adenosylmethionine. J Biol Chem. 1981;256(19):10028–32.
157. Mudd SH, Brosnan JT, Brosnan ME, Jacobs RL, Stabler SP, Allen RH, et al. Methyl balance and transmethylation fluxes in humans. Am J Clin Nutr. 2018;85(1):19–25.
158. Su X, Wellen KE, Rabinowitz JD. Metabolic control of methylation and acetylation. Curr Opin Chem Biol. 2016;30:52–60.
159. Wright AJA, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: Potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr. 2007;98(4):667–75.
160. Hou Z, Matherly LH. Biology of the major facilitative folate transporters SLC19A1 and SLC46A1. 1st ed. Vol. 73, Current Topics in Membranes. Elsevier Inc.; 2014. 175–204 p.
161. Kim DW, Huang T, Schirch D, Schirch V. Properties of tetrahydropteroylpentaglutamate bound to 10-formyltetrahydrofolate dehydrogenase. Biochemistry. 1996;35(49):15772–83.
162. Freemantle SJ, Taylor SM, Krystal G, Moran RG. Upstream Organization of and Multiple Transcripts from the Human Folylpoly-gamma-glutamate Synthetase Gene. J Biol Chem. 1995;270(16):9579–84.
163. Anderson DD, Quintero CM, Stover PJ. Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria. Proc Natl Acad Sci. 2011;108(37):15163–8.
164. de Koning TJ, Snell K, Duran M, Berger R, Poll-The B-T, Surtees R. L-Serine in disease and development. Biochem J. 2003;371(3):653–61.
165. Kalhan SC, Hanson RW. Resurgence of serine: An often neglected but indispensable amino acid. J Biol Chem. 2012;287(24):19786–91.
166. Chaneton B, Hillmann P, Zheng L, Martin ACL, Maddocks ODK, Chokkathukalam A, et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature. 2012;491(7424):458–62.
167. Ye J, Mancuso A, Tong X, Ward PS, Fan J, Rabinowitz JD, et al. Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. Proc Natl Acad Sci. 2012;109(18):6904–9.
168. Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nat Genet. 2011;43(9):869–74.
169. Snell K. Enzymes of serine metabolism in normal, developing and neoplastic rat tissues. Adv Enzyme Regul. 1984;22:325–400.
170. Snell K, Weber G. Enzymic imbalance in serine metabolism in rat hepatomas. Biochem J. 1986;233(2):617–20.
171. Snell K, Natsumeda Y, Weber G. The modulation of serine metabolism in hepatoma 3924A during different phases of cellular proliferation in culture. Biochem J. 1987;245(2):609–12.
172. Pollari S, Käkönen SM, Edgren H, Wolf M, Kohonen P, Sara H, et al. Enhanced serine production by bone metastatic breast cancer cells stimulates osteoclastogenesis. Breast Cancer Res Treat. 2011;125(2):421–30.
173. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab. 2016;25(2017).
174. Pike ST, Rajendra R, Artzt K, Appling DR. Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. J Biol Chem. 2010;285(7):4612–20.
175. Scrimgeour KG, Huennekens FM. Occurrence of a DPN-linked, N5,N10-methylene tetrahydrofolic dehydrogenase in Ehrlich ascites tumor cells. Biochem Biophys Res Commun. 1960;2(3):230–3.
176. Mejia NR, MacKenzie RE. NAD-dependent methylenetetrahydrofolate dehydrogenase is expressed by immortal cells.

J Biol Chem. 1985;260(27):14616–20.
177. Patel H, Di Pietro E, MacKenzie RE. Mammalian fibroblasts lacking mitochondrial NAD+-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase are glycine auxotrophs. J Biol Chem. 2003;278(21):19436.
178. Christensen KE, Mirza IA, Berghuis AM, MacKenzie RE. Magnesium and phosphate ions enable NAD binding to methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase. J Biol Chem. 2005;280(40):34316–23.
179. Nilsson R, Nicolaidou V, Koufaris C. Mitochondrial MTHFD isozymes display distinct expression, regulation, and association with cancer. Gene. 2019;716(May):144032.
180. Rowe PB, Lewis GP. Mammalian Folate Metabolism. Regulation of Folate Interconversion Enzymes. Biochemistry. 1973;12(10):1962–9.
181. Ducker GS, Chen L, Morscher RJ, Ghergurovich JM, Esposito M, Teng X, et al. Reversal of Cytosolic One-Carbon Flux Compensates for Loss of the Mitochondrial Folate Pathway. Cell Metab. 2016;23(6):1140–53.
182. Tong X, Zhao F, Thompson CB. The molecular determinants of de novo nucleotide biosynthesis in cancer cells. Curr Opin Genet Dev. 2009;19(1):32–7.
183. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. In Vivo Kinetics of Thymidylate Synthetase Inhibition in 5-FU Sensitive and Resistance Murine Colon Adneocarcinomas. Cancer Res. 1982;42(February):450–6.
184. Pinedo HM, Peters GFJ. Fluorouracil: Biochemistry and Pharmacology. J Clin Oncol. 1988;6(10):1653–64.
185. Diasio RB, Harris BE. Clinical Pharmacology of 5-Fluorouracil. Clin Pharmacokinet. 1989;16(4):215–37.
186. Widemann BC, Balis FM, Godwin KS, McCully C, Adamson PC. The plasma pharmacokinetics and cerebrospinal fluid penetration of the thymidylate synthase inhibitor raltitrexed (Tomudex(TM)) in a nonhuman primate model. Cancer Chemother Pharmacol. 1999;44(6):439–43.
187. An S, Kumar R, Sheets ED, Benkovic SJ. Reversible Compartmentalization of de Novo Purine Biosynthetic Complexes in Living Cells. Science (80-). 2008;320(5872):103–6.
188. French JB, Pedley AM, Kim D, Chan CY, Hu H, Zhao H, et al. Spatial colocalization and functional link of purinosomes with mitochondria. Science (80-). 2016;351(6274):733–7.
189. Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD. Quantitative flux analysis reveals folate-dependent NADPH production. Nature. 2014;510(7504):298–302.
190. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, et al. Oxidative stress inhibits distant metastasis by human melanoma cells. Nature. 2015;527(7577):186–91.
191. Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. Am J Clin Nutr. 2005;81(5):1213–7.
192. Iskandar BJ, Rizk E, Meier B, Hariharan N, Bottiglieri T, Finnell RH, et al. Folate regulation of axonal regeneration in the rodent central nervous system through DNA methylation. J Clin Invest. 2010;120(5):1603–16.
193. Roth C, Magnus P, Schjølberg S, Reichborn-Kjennerud T, Susser E. Folic Acid Supplements in Pregnancy and Severe Language Delay in Children. JAMA J Am Med Assoc. 2013;306(14):1566–73.
194. Wallingford JB, Niswander LA, Shaw GM, Finnell RH. The Continuing Challenge of Understanding, Preventing, and Treating Neural Tube Defects. Science (80-). 2013;339(6123):1011–1011.
195. MacFarlane AJ, Perry CA, Girnary HH, Gao D, Allen RH, Stabler SP, et al. Mthfd1 is an essential gene in mice and alters biomarkers of impaired one-carbon metabolism. J Biol Chem. 2009;284(3):1533–9.
196. Beaudin AE, Abarinov E V., Noden DM, Perry CA, Chu S, Stabler SP, et al. Shmt1 and de novo thymidylate biosynthesis underlie folate-responsive neural tube defects in mice. Am J Clin Nutr. 2011;93(4):789–98.
197. Narisawa A, Komatsuzaki S, Kikuchi A, Niihori T, Aoki Y, Fujiwara K, et al. Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans. Hum Mol Genet. 2012;21(7):1496–503.
198. Momb J, Lewandowski JP, Bryant JD, Fitch R, Surman DR, Vokes SA, et al. Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. Proc Natl Acad Sci. 2012;110(2):549–54.
199. Mehrmohamadi M, Liu X, Shestov AA, Locasale JW. Characterization of the Usage of the Serine Metabolic Network in Human Cancer. Cell Rep. 2014;9(4):1507–19.
200. Ben-Sahra I, Hoxhaj G, Ricoult SJH, Asara JM, Manning BD. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science (80-). 2016;351(6274):728–33.
201. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960–76.
202. Ahuja P, Zhao P, Angelis E, Ruan H, Korge P, Olson A, et al. Myc controls transcriptional regulation of cardiac metabolism and mitochondrial biogenesis in response to pathological stress in mice. J Clin Invest. 2010;120(5):1494.
203. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang X-Y, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci. 2008;105(48):18782–7.
204. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, et al. C-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009;458(7239):762–5.
205. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci. 2010;107(19):8788–93.
206. Moran DM, Trusk PB, Pry K, Paz K, Sidransky D, Bacus SS. KRAS Mutation Status Is Associated with Enhanced Dependency on Folate Metabolism Pathways in Non-Small Cell Lung Cancer Cells. Mol Cancer Ther. 2014;13(6):1611–24.
207. Nilsson R, Jain M, Madhusudhan N, Sheppard NG, Strittmatter L, Kampf C, et al. Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer. Nat Commun. 2014;5:1–10.
208. Lehtinen L, Ketola K, Mäkelä R, Mpindi J-P, Viitala M, Kallioniemi O, et al. High-throughput RNAi screening for novel modulators of vimentin expression identifies MTHFD2 as a regulator of breast cancer cell migration and invasion. Oncotarget. 2013;4(1):48–63.
209. Liu F, Liu Y, He C, Tao L, He X, Song H, et al. Increased MTHFD2 expression is associated with poor prognosis in breast cancer. Tumor Biol. 2014;35(9):8685–90.
210. Pikman Y, Puissant A, Alexe G, Furman A, Chen LM, Frumm SM, et al. Targeting MTHFD2 in acute myeloid

leukemia. J Exp Med. 2016;213(7):1285–306.
211. Gustafsson Sheppard N, Jarl L, Mahadessian D, Strittmatter L, Schmidt A, Madhusudan N, et al. The folate-coupled enzyme MTHFD2 is a nuclear protein and promotes cell proliferation. Sci Rep. 2015;5:1–11.
212. Koufaris C, Nilsson R. Protein interaction and functional data indicate MTHFD2 involvement in RNA processing and translation. Cancer Metab. 2018;6(1):1–14.
213. Pardee AB, Prem veer Reddy G. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc Natl Acad Sci U S A. 1980;77(6):3312–6.
214. Anderson DD, Stover PJ. SHMT1 and SHMT2 are functionally redundant in nuclear de novo thymidylate biosynthesis. PLoS One. 2009;4(6).
215. Anderson DD, Woeller CF, Chiang EP, Shane B, Stover PJ. Serine hydroxymethyltransferase anchors de Novo thymidylate synthesis pathway to nuclear lamina for DNA synthesis. J Biol Chem. 2012;287(10):7051–62.
216. Field MS, Kamynina E, Agunloye OC, Liebenthal RP, Lamarre SG, Brosnan ME, et al. Nuclear enrichment of folate cofactors and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protect de novo thymidylate biosynthesis during folate deficiency. J Biol Chem. 2014;289(43):29642–50.
217. Field MS, Kamynina E, Stover PJ. MTHFD1 regulates nuclear de novo thymidylate biosynthesis and genome stability. Biochimie. 2016;126:27–30.
218. Koufaris C, Gallage S, Yang T, Lau CH, Valbuena GN, Keun HC. Suppression of MTHFD2 in MCF-7 breast cancer cells increases glycolysis, dependency on exogenous glycine, and sensitivity to folate depletion. J Proteome Res. 2016;15(8):2618–25.
219. Glasauer A, Steckel M, Haegebarth A, Bauser M, Toschi L. Targeting the one-carbon metabolism protein MTHFD2 for cancer therapy: Exploiting the unique redox status of cancer cells. Cancer Res. 2016;76(14 Supplement):3790.
220. Kawai J, Toki T, Ota M, Inoue H, Takata Y, Asahi T, et al. Discovery of a Potent, Selective, and Orally Available MTHFD2 Inhibitor (DS18561882) with in Vivo Antitumor Activity. J Med Chem. 2019;
221. Wei Y, Liu P, Li Q, Du J, Chen Y, Wang Y, et al. The effect of MTHFD2 on the proliferation and migration of colorectal cancer cell lines. Onco Targets Ther. 2019;12:6361–70.
222. Ju HQ, Lu YX, Chen DL, Zuo ZX, Liu ZX, Wu QN, et al. Modulation of Redox Homeostasis by Inhibition of MTHFD2 in Colorectal Cancer: Mechanisms and Therapeutic Implications. J Natl Cancer Inst. 2019;111(6):584–96.
223. Yan Y, Zhang D, Lei T, Zhao C, Han J, Cui J, et al. MicroRNA-33a-5p suppresses colorectal cancer cell growth by inhibiting MTHFD2. Clin Exp Pharmacol Physiol. 2019;46(10):928–36.
224. Nishimura T, Nakata A, Chen X, Nishi K, Meguro-Horike M, Sasaki S, et al. Cancer stem-like properties and gefitinib resistance are dependent on purine synthetic metabolism mediated by the mitochondrial enzyme MTHFD2. Oncogene. 2018;2464–81.
225. Yu C, Yang L, Cai M, Zhou F, Xiao S, Li Y, et al. Down-regulation of MTHFD2 inhibits NSCLC progression by suppressing cycle-related genes. J Cell Mol Med. 2020;24(2):1568–77.
226. Chan CH, Wu CY, Dubey NK, Wei HJ, Lu JH, Mao S, et al. Modulating redox homeostasis and cellular reprogramming through inhibited methylenetetrahydrofolate dehydrogenase 2 enzymatic activities in lung cancer. Aging (Albany NY). 2020;12(18):17930–47.
227. Gu Y, Si J, Xiao X, Tian Y, Yang S. MiR-92a inhibits proliferation and induces apoptosis by regulating methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) expression in acute myeloid leukemia. Oncol Res. 2017;
228. Li SM, Zhao YQ, Hao YL, Liang YY. Upregulation of miR-504-3p is associated with favorable prognosis of acute myeloid leukemia and may serve as a tumor suppressor by targeting MTHFD2. Eur Rev Med Pharmacol Sci. 2019;23(3):1203–13.
229. Lin H, Huang B, Wang H, Liu X, Hong Y, Qiu S, et al. MTHFD2 overexpression predicts poor prognosis in renal cell carcinoma and is associated with cell proliferation and vimentin-modulated migration and invasion. Cell Physiol Biochem. 2018;51(2):991–1000.
230. Green NH, Galvan DL, Badal SS, Chang BH, LeBleu VS, Long J, et al. MTHFD2 links RNA methylation to metabolic reprogramming in renal cell carcinoma. Oncogene. 2019;
231. Xu T, Zhang K, Shi J, Huang B, Wang X, Qian K, et al. MicroRNA-940 inhibits glioma progression by blocking mitochondrial folate metabolism through targeting of MTHFD2. Am J Cancer Res. 2019;9(2):250–69.
232. Cheung CHY, Hsu CL, Tsuei CY, Kuo TT, Huang CT, Hsu WM, et al. Combinatorial targeting of MTHFD2 and PAICS in purine synthesis as a novel therapeutic strategy. Cell Death Dis. 2019;
233. Liu X, Huang Y, Jiang C, Ou H, Guo B, Liao H, et al. Methylenetetrahydrofolate dehydrogenase 2 overexpression is associated with tumor aggressiveness and poor prognosis in hepatocellular carcinoma. Dig Liver Dis. 2016;48(8):953.
234. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461(7267):1071–8.
235. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability an evolving hallmark of cancer. Nat Rev Mol Cell Biol. 2010;11(3):220–8.
236. Salk JJ, Fox EJ, Loeb LA. Mutational Heterogeneity in Human Cancers: Origin and Consequences. Annu Rev Pathol Mech Dis. 2009;5(1):51–75.
237. Bartkova J, Horejsí Z, Koed K, A, Krämer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005;434(7035):864–87.
238. Gorgoulis VG, Vassiliou L-VF, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005;907–13.
239. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008;8(3):193–204.
240. Forment J V., O'Connor MJ. Targeting the replication stress response in cancer. Pharmacol Ther. 2018;188:155–67.
241. Lecona E, Fernandez-Capetillo O. Targeting ATR in cancer. Nat Rev Cancer. 2018;18:586–95.
242. Hoeijmakers JHJ. Genome maintenance mechanisms are critical for preventing cancer as well as other aging-associated diseases. Mech Ageing Dev. 2007;128(7–8):460–2.
243. Boyer AS, Grgurevic S, Cazaux C, Hoffmann JS. The human specialized DNA polymerases and non-B DNA: Vital relationships to preserve genome integrity. J Mol Biol. 2013;425(23):4767–81.

244. Bester AC, Roniger M, Oren YS, Im MM, Sarni D, Chaoat M, et al. Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell. 2011;145(3):435–46.
245. Hu CM, Yeh MT, Tsao N, Chen CW, Gao QZ, Chang CY, et al. Tumor Cells Require Thymidylate Kinase to Prevent dUTP Incorporation during DNA Repair. Cancer Cell. 2012;22(1):36–50.
246. Stover PJ, Weiss RS. Sensitizing Cancer Cells: Is It Really All about U? Cancer Cell. 2012;22(1):3–4.
247. Lane AN, Fan TWM. Regulation of mammalian nucleotide metabolism and biosynthesis. Nucleic Acids Res. 2015;43(4):2466–85.
248. Mathews CK. Deoxyribonucleotide metabolism, mutagenesis and cancer. Nat Rev Cancer. 2015;15(9):528–39.
249. Traut TW. Physiological concentrations of purines and pyrimidines. Mol Cell Biochem. 1994;140(1):1–22.
250. Rahman L, Voeller D, Rahman M, Lipkowitz S, Allegra C, Barrett JC, et al. Thymidylate synthase as an oncogene: A novel role for an essential DNA synthesis enzyme. Cancer Cell. 2004;5(4):341–51.
251. Bertino JR, Banerjee D. Thymidylate synthase as an oncogene? Cancer Cell. 2004;(April):301–2.
252. Xu X, Page JL, Surtees JA, Liu H, Lagedrost S, Lu Y, et al. Broad overexpression of ribonucleotide reductase genes in mice specifically induces lung neoplasms. Cancer Res. 2008;68(8):2652–60.
253. Fukushima M, Fujioka A, Uchida J, Nakagawa F, Takechi T. Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts in vivo. Eur J Cancer. 2001;37(13):1681–7.
254. Zhou L, Xia D, Zhu J, Chen Y, Chen G, Mo R, et al. Enhanced expression of IMPDH2 promotes metastasis and advanced tumor progression in patients with prostate cancer. Clin Transl Oncol. 2014;16(10):906–13.
255. Barnes BJ, Eakin AE, Izydore RA, Hall IH. Implications of selective type II IMP dehydrogenase (IMPDH) inhibition by the 6-ethoxycarbonyl-3,3-disubstituted-1,5-diazabicyclo[3.1.0]hexane-2,4-diones on tumor cell death. Biochem Pharmacol. 2001;62(1):91–100.
256. Cong X, Lu C, Huang X, Yang D, Cui X, Cai J, et al. Increased expression of glycinamide ribonucleotide transformylase is associated with a poor prognosis in hepatocellular carcinoma, and it promotes liver cancer cell proliferation. Hum Pathol. 2014;45(7):1370–8.
257. Wang L, Desmoulin SK, Cherian C, Polin L, White K, Kushner J, et al. Synthesis, biological, and antitumor activity of a highly potent 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate inhibitor with proton-coupled folate transporter and folate receptor selectivity over the reduced folate carrier that inhibits β-gl. J Med Chem. 2011;54(20):7150–64.
258. Wilson PM, LaBonte MJ, Lenz H-J, Mack PC, Ladner RD. Inhibition of dUTPase Induces Synthetic Lethality with Thymidylate Synthase-Targeted Therapies in Non-Small Cell Lung Cancer. Mol Cancer Ther. 2011;11(3):616–28.
259. Baumann P, Mandl-Weber S, Volkl A, Adam C, Bumeder I, Oduncu F, et al. Dihydroorotate dehydrogenase inhibitor A771726 (leflunomide) induces apoptosis and diminishes proliferation of multiple myeloma cells. Mol Cancer Ther. 2009;8(2):366–75.
260. Ladds MJGW, Van Leeuwen IMM, Drummond CJ, Chu S, Healy AR, Popova G, et al. A DHODH inhibitor increases p53 synthesis and enhances tumor cell killing by p53 degradation blockage. Nat Commun. 2018;9(1).
261. Miltenberger RJ, Sukow KA, Farnham PJ. An E-box-mediated increase in cad transcription at the G1/S-phase boundary is suppressed by inhibitory c-Myc mutants. Mol Cell Biol. 2015;15(5):2527–35.
262. Valvezan AJ, Turner M, Belaid A, Lam HC, Miller SK, McNamara MC, et al. mTORC1 Couples Nucleotide Synthesis to Nucleotide Demand Resulting in a Targetable Metabolic Vulnerability. Cancer Cell. 2017;32(5):624-638.e5.
263. Cunningham JT, Moreno M V., Lodi A, Ronen SM, Ruggero D. Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer. Cell. 2014;157(5):1088–103.
264. Ben-Sahra I, Howell JJ, Asara JM, Manning BD. Stimulation of de Novo Pyrimidine Synthesis by Growth Signaling Through mTOR and S6K1. Science (80-). 2013;339(6125):1323–8.
265. Robitaille AM, Christen S, Shimobayashi M, Cornu M, Fava LL, Moes S, et al. Quantitative Phosphoproteomics Reveal mTORC1 Activates de Novo Pyrimidine Synthesis. Science (80-). 2013;339(6125):1320–3.
266. Cohen SS, Barner HD. Studies on Unbalanced Growth in Escherichia Coli. Proc Natl Acad Sci. 1954;40(10):885–93.
267. Zhang W, Tan S, Paintsil E, Dutschman GE, Gullen EA, Chu E, et al. Analysis of deoxyribonucleotide pools in human cancer cell lines using a liquid chromatography coupled with tandem mass spectrometry technique. Biochem Pharmacol. 2011;82(4):411–7.
268. Krokan HE, Bjørås M. Base Excision Repair. Cold Spring Harb Perspect Biol. 2013;5(4).
269. Davidson MB, Katou Y, Keszthelyi A, Sing TL, Xia T, Ou J, et al. Endogenous DNA replication stress results in expansion of dNTP pools and a mutator phenotype. EMBO J. 2012;31(4):895–907.
270. Canman CE, Lawrence TS, Shewach DS, Tang HY, Maybaum J. Resistance to Fluorodeoxyuridine-induced DNA Damage and Cytotoxicity Correlates with an Elevation of Deoxyuridine Triphosphatase Activity and Failure to Accumulate Deoxyuridine Triphosphate. Cancer Res. 1993;53(21):5219–24.
271. Ladner RD, Lynch FJ, Groshen S, Xiong YP, Sherrod A, Caradonna SJ, et al. dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: Association with survival and response to 5-fluorouracil in colorectal cancer. Cancer Res. 2000;60(13):3493–503.
272. Wilson PM, Danenberg P V., Johnston PG, Lenz HJ, Ladner RD. Standing the test of time: Targeting thymidylate biosynthesis in cancer therapy. Nat Rev Clin Oncol. 2014;11(5):282–98.
273. Ladner RD, McNulty DE, Carr SA, Roberts GD, Caradonna SJ. Characterization of distinct nuclear and mitochondrial forms of human dUTPase. J Biol Chem. 1996;271(13):7745–51.
274. Niida H, Katsuno Y, Sengoku M, Shimada M, Yukawa M, Ikura M, et al. Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. Genes Dev. 2010;24(4):333–8.
275. MacFarlane AJ, Anderson DD, Flodby P, Perry CA, Allen RH, Stabler SP, et al. Nuclear localization of de Novo thymidylate biosynthesis pathway is required to prevent uracil accumulation in DNA. J Biol Chem. 2011;286(51):44015–22.
276. Reichard P. Interactions Between Deoxyribonucleotide And DNA Synthesis. Annu Rev Biochem. 2002;57(1):349.

277. Kunz BA, Kohalmi SE. Modulation of mutagenesis by deoxyribonucleotide levels. Annu Rev Genet. 1991;25:339.
278. Chan KL, Palmai-Pallag T, Ying S, Hickson ID. Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. Nat Cell Biol. 2009;11(6):753–60.
279. Burrell RA, McClelland SE, Endesfelder D, Groth P, Weller MC, Shaikh N, et al. Replication stress links structural and numerical cancer chromosomal instability. Nature. 2013;494(7438):492–6.
280. Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nat Cell Biol. 2014;16(1):2–9.
281. Minocherhomji S, Ying S, Bjerregaard VA, Bursomanno S, Aleliunaite A, Wu W, et al. Replication stress activates DNA repair synthesis in mitosis. Nature. 2015;528(7581):286–90.
282. Giannattasio M, Branzei D. S-phase checkpoint regulations that preserve replication and chromosome integrity upon dNTP depletion. Cell Mol Life Sci. 2017;74(13):2361–80.
283. Zhang BN, Bueno Venegas A, Hickson ID, Chu WK. DNA replication stress and its impact on chromosome segregation and tumorigenesis. Semin Cancer Biol. 2018;55(April 2018):61–9.
284. Branzei D, Foiani M. Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol. 2010;11(3):208.
285. Shen C, Lancaster CS, Shi B, Guo H, Thimmaiah P, Bjornsti M-A. TOR Signaling Is a Determinant of Cell Survival in Response to DNA Damage. Mol Cell Biol. 2007;27(20):7007–17.
286. Selvarajah J, Elia A, Carroll VA, Moumen A. DNA damage-induced S and G2/M cell cycle arrest requires mTORC2-dependent regulation of Chk1. Oncotarget. 2015;6(1):19–21.
287. Saldivar JC, Cortez D, Cimprich KA. The essential kinase ATR: Ensuring faithful duplication of a challenging genome. Nat Rev Mol Cell Biol. 2017;18(10):622–36.
288. D'Angiolella V, Donato V, Forrester FM, Jeong YT, Pellacani C, Kudo Y, et al. Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. Cell. 2012;149(5):1023–34.
289. Cuyas E, Corominas-Faja B, Joven J, Menendez JA. Cell Cycle Regulation by the Nutrient-Sensing Mammalian Target of Rapamycin (mTOR) Pathway. In: Cell Cycle Control Methods in Molecular Biology (Methods and Protocols). 2014. p. 713–6.
290. Medema RH, Kops GJPL, Bos JL, Burgering BMT. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature. 2000;404(April):782–7.
291. Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C, Blenis J. mTOR Controls Cell Cycle Progression through Its Cell Growth Effectors S6K1 and 4E-BP1/Eukaryotic Translation Initiation Factor 4E. Mol Cell Biol. 2003;24(1):200–16.
292. Averous J, Fonseca BD, Proud CG. Regulation of cyclin D1 expression by mTORC1 signaling requires eukaryotic initiation factor 4E-binding protein 1. Oncogene. 2008;27(8):1106–13.
293. Hong F, Larrea MD, Doughty C, Kwiatkowski DJ, Squillace R, Slingerland JM. mTOR-Raptor Binds and Activates SGK1 to Regulate p27 Phosphorylation. Mol Cell. 2008;30(6):701–11.
294. Oka K, Ohya-Shimada W, Mizuno S, Nakamura T. Up-regulation of cyclin-E1 via proline-mTOR pathway is responsible for HGF-mediated G1/S progression in the primary culture of rat hepatocytes. Biochem Biophys Res Commun. 2013;435(1):120–5.
295. Mondesire WH, Jian W, Zhang H, Ensor J, Hung MC, Mills GB, et al. Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells. Clin Cancer Res. 2004;10(20):7031–42.
296. Shen C, Oswald D, Phelps D, Cam H, Pelloski CE, Pang Q, et al. Regulation of FANCD2 by the mTOR pathway contributes to the resistance of cancer cells to DNA double-strand breaks. Cancer Res. 2013;73(11):3393–401.
297. Steelman LS, Navolanic PM, Sokolosky ML, Taylor JR, Lehmann BD, Chappell WH, et al. Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTOR inhibitors. Oncogene. 2008;27(29):4086–95.
298. Zhou X, Liu W, Hu X, Dorrance A, Garzon R, Houghton PJ, et al. Regulation of CHK1 by mTOR contributes to the evasion of DNA damage barrier of cancer cells. Sci Rep. 2017;7(1):1–13.
299. Silvera D, Ernlund A, Arju R, Connolly E, Volta V, Wang J, et al. mTORC1 and -2 Coordinate Transcriptional and Translational Reprogramming in Resistance to DNA Damage and Replicative Stress in Breast Cancer Cells. Mol Cell Biol. 2017;37(5).
300. Barnieh FM, Loadman PM, Falconer RA. Progress towards a clinically-successful ATR inhibitor for cancer therapy. Curr Res Pharmacol Drug Discov. 2021;2(January):100017.
301. Pilié PG, Tang C, Mills GB, Yap TA. State-of-the-art strategies for targeting the DNA damage response in cancer. Nature Reviews Clinical Oncology. 2019.
302. Ciccia A, Elledge SJ. The DNA Damage Response: Making It Safe to Play with Knives. Mol Cell. 2010;40(2):179.
303. Ward JF. Radiation-induced strand breakage in DNA. Basic Life Sci. 1975;5B:371–472.
304. Ho KSY, Mortimer RK. Two mutations which confer temperature-sensitive radiation sensitivity in the yeast Saccharomyces cerevisiae. Mutat Res. 1975;33:157–64.
305. Radford IR. The level of induced DNA double-Strand breakage correlates with cell killing after x-irradiation. Int J Radiat Biol. 1985;48(1):45–54.
306. Wright WD, Shah SS, Heyer WD. Homologous recombination and the repair of DNA double-strand breaks. J Biol Chem. 2018;293(27):10524–35.
307. Ceccaldi R, Rondinelli B, D 'andrea AD. Repair Pathway Choices and Consequences at the Double- Strand Break Mechanisms of DNA DSB Repair. Trends Biochem Sci. 2016;26(1):52–64.
308. Scully R, Panday A, Elango R, Willis NA. DNA double-strand break repair-pathway choice in somatic mammalian cells. Nat Rev Mol Cell Biol. 2019;20(11):698–714.
309. Hustedt N, Durocher D. The control of DNA repair by the cell cycle. Nat Cell Biol. 2017;19(1):1–9.
310. Chapman JR, Taylor MRG, Boulton SJ. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Mol Cell. 2012;47(4):497–510.
311. Blackford AN, Jackson SP. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Molecular Cell. 2017.

312. Bartek J, Bartkova J, Lukas J. DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene. 2007;26(56):7773–9.
313. Lord CJ, Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. Science (80-). 2017;355(March):1152–8.
314. Puigvert JC, Sanjiv K, Helleday T. Targeting DNA repair, DNA metabolism and replication stress as anti-cancer strategies. FEBS J. 2016;283(2):232–45.
315. Turgeon MO, Perry NJS, Poulogiannis G. DNA damage, repair, and cancer metabolism. Front Oncol. 2018;8(FEB).
316. Moretton A, Loizou JI. Interplay between cellular metabolism and the dna damage response in cancer. Cancers (Basel). 2020;12(8):1–29.
317. Sizemore ST, Zhang M, Cho JH, Sizemore GM, Hurwitz B, Kaur B, et al. Pyruvate kinase M2 regulates homologous recombination-mediated DNA double-strand break repair. Cell Res. 2018;28(11):1090–102.
318. Lahiguera Á, Hyroššová P, Figueras A, Garzón D, Moreno R, Soto-Cerrato V, et al. Tumors defective in homologous recombination rely on oxidative metabolism: relevance to treatments with PARP inhibitors. EMBO Mol Med. 2020;12(6):1–23.
319. Gustafsson NMS, Färnegårdh K, Bonagas N, Huguet Ninou A, Groth P, Wiita E, et al. Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination. Nat Commun. 2018;9(1).
320. Burkhalter MD, Roberts SA, Havener JM, Ramsden DA. Activity of ribonucleotide reductase helps determine how cells repair DNA double strand breaks. DNA Repair (Amst). 2009;8(11):1258–63.
321. Fu S, Li Z, Xiao L, Hu W, Zhang L, Xie B, et al. Glutamine Synthetase Promotes Radiation Resistance via Facilitating Nucleotide Metabolism and Subsequent DNA Damage Repair. Cell Rep. 2019;28(5):1136-1143.e4.
322. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO J. 2011;30(3):546–55.
323. Qu J, Sun W, Zhong J, Lv H, Zhu M, Xu J, et al. Phosphoglycerate mutase 1 regulates dNTP pool and promotes homologous recombination repair in cancer cells. J Cell Biol. 2017;216(2):409–24.
324. Xia L, Qin K, Wang XR, Wang XL, Zhou AW, Chen GQ, et al. Pyruvate kinase M2 phosphorylates H2AX and promotes genomic instability in human tumor cells. Oncotarget. 2017;8(65):109120–34.
325. Lee CY, Su GC, Huang WY, Ko MY, Yeh HY, Chang GD, et al. Promotion of homology-directed DNA repair by polyamines. Nat Commun. 2019;10(1):1–11.
326. Geck RC, Foley JR, Stewart TM, Asara JM, Casero RA, Toker A. Inhibition of the polyamine synthesis enzyme ornithine decarboxylase sensitizes triple-negative breast cancer cells to cytotoxic chemotherapy. J Biol Chem. 2020;295(19):6263–77.
327. Zaidi N, Swinnen J V., Smans K. ATP-citrate lyase: A key player in cancer metabolism. Cancer Res. 2012;72(15):3709–14.
328. Sivanand S, Rhoades S, Jiang Q, Lee J V., Benci J, Zhang J, et al. Nuclear Acetyl-CoA Production by ACLY Promotes Homologous Recombination. Mol Cell. 2017;67(2):252-265.e6.
329. Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui T V., Cross JR, Thompson CB. ATP-citrate lyase links cellular metabolism to histone acetylation. Science (80-). 2009;324(5930):1076–80.
330. Tang J, Cho NW, Cui G, Manion EM, Shanbhag NM, Botuyan MV, et al. Acetylation limits 53BP1 association with damaged chromatin to promote homologous recombination. Nat Struct Mol Biol. 2013;20(3):317–25.
331. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;19(1):17–30.
332. Sulkowski PL, Oeck S, Dow J, Economos NG, Mirfakhraie L, Liu Y, et al. Oncometabolites suppress DNA repair by disrupting local chromatin signalling. Vol. 582, Nature. Springer US; 2020. 586–591 p.
333. Sulkowski PL, Sundaram RK, Oeck S, Corso CD, Liu Y, Noorbakhsh S, et al. Krebs-cycle-deficient hereditary cancer syndromes are defined by defects in homologous-recombination DNA repair. Nat Genet. 2018;50(8):1086–92.
334. Chen L, Xiong Y. Tumour metabolites hinder DNA repair. Nature. 2020;582:492–4.
335. Jiang Y, Qian X, Shen J, Wang Y, Li X, Liu R, et al. Local generation of fumarate promotes DNA repair through inhibition of histone H3 demethylation. Nat Cell Biol. 2015;17(9):1158–68.
336. Leshets M, Silas YBH, Lehming N, Pines O. Fumarase: From the TCA Cycle to DNA Damage Response and Tumor Suppression. Front Mol Biosci. 2018;5(July):1–10.
337. Efimova E V., Appelbe OK, Ricco N, Lee SSY, Liu Y, Wolfgeher DJ, et al. O-GlcN acylation enhances double-strand break repair, promotes cancer cell proliferation, and prevents therapy-induced senescence in irradiated tumors. Mol Cancer Res. 2019;17(6):1338–50.
338. Ward JF. Some Biochemical Consequences of the Spatial Distribution of Ionizing Radiation-Produced Free Radicals. Radiat Res. 1981;86(2):185–95.
339. Chapman JD, Reuvers AP, Borsa J, Greenstock CL. Chemical Radioprotection and Radiosensitization of Mammalian Cells Growing in Vitro. Radiat Res. 2012;178(2):AV214–22.
340. Tapio S, Jacob V. Radioadaptive response revisited. Radiat Environ Biophys. 2007;46(1):1–12.
341. Jeggo P, Lavin MF. Cellular radiosensitivity: How much better do we understand it? Int J Radiat Biol. 2009;85(12):1061–81.
342. Van Der Schans GP, Bleichrodt JF, Blok J. Contribution of various types of damage to inactivation of a biologically-active double-stranded circular DNA by gamma-radiation. Int J Radiat Biol. 1973;23(2):133–50.
343. Lawrence TS, Blackstock AW, McGinn C. The mechanism of action of radiosensitization of conventional chemotherapeutic agents. Semin Radiat Oncol. 2003;13(1):13–21.
344. Biau J, Chautard E, Verrelle P, Dutreix M. Altering DNA repair to improve radiation therapy: Specific and multiple pathway targeting. Front Oncol. 2019;9(SEP):1–10.
345. Harrison LB, Chadha M, Hill RJ, Hu K, Shasha D. Impact of Tumor Hypoxia and Anemia on Radiation Therapy Outcomes. Oncologist. 2002;7(6):492–508.
346. Gainer JL, Sheehan JP, Larner JM, Jones DR. Trans sodium crocetinate with temozolomide and radiation therapy for glioblastoma multiforme. J Neurosurg. 2017;126(2):460–6.
347. Saksø M, Jensen K, Andersen M, Hansen CR, Eriksen JG, Overgaard J. DAHANCA 28: A phase I/II feasibility study

of hyperfractionated, accelerated radiotherapy with concomitant cisplatin and nimorazole (HART-CN) for patients with locally advanced, HPV/p16-negative squamous cell carcinoma of the oropharynx, hypopharynx, lary. Radiother Oncol. 2020;148:65–72.
348. Bonvalot S, Rutkowski PL, Thariat J, Carrère S, Ducassou A, Sunyach MP, et al. NBTXR3, a first-in-class radioenhancer hafnium oxide nanoparticle, plus radiotherapy versus radiotherapy alone in patients with locally advanced soft-tissue sarcoma (Act.In.Sarc): a multicentre, phase 2–3, randomised, controlled trial. Lancet Oncol. 2019;20(8):1148–59.
349. Unger EC, Lickliter JD, Ruben J, Jennens R, Kichenadasse G, Gzell C, et al. A phase Ib/II clinical trial of a novel oxygen therapeutic in chemoradiation of glioblastoma. J Clin Oncol. 2017;35(15 Supplement):2561–2561.
350. Wang G, Wang JJ, Yin PH, Xu K, Wang YZ, Shi F, et al. New strategies for targeting glucose metabolism–mediated acidosis for colorectal cancer therapy. J Cell Physiol. 2018;234(1):348–68.
351. Lin J, Xia L, Liang J, Han Y, Wang H, Oyang L, et al. The roles of glucose metabolic reprogramming in chemo- and radio-resistance. J Exp Clin Cancer Res. 2019;38(1):1–13.
352. Bartrons R, Rodríguez-García A, Simon-Molas H, Castaño E, Manzano A, Navarro-Sabaté À. The potential utility of PFKFB3 as a therapeutic target. Expert Opin Ther Targets. 2018;22(8):659–74.
353. Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, et al. Inhibition of glycolysis in cancer cells: A novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. 2005;65(2):613–21.
354. Liu H, Hu YP, Savaraj N, Priebe W, Lampidis TJ. Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry. 2001;40(18):5542–7.
355. Lu L, Chen Y, Zhu Y. The molecular basis of targeting PFKFB3 as a therapeutic strategy against cancer. Oncotarget. 2017;8(37):62793–802.
356. Chin L, DePinho# RA, Tam A, Pomerantz J, Wong M, Holash J, et al. Essential role for oncogenic Ras in tumour maintenance. Nature. 1999;400(6743):468–72.
357. Huettner CS, Zhang P, Van Etten RA, Tenen DG. Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat Genet. 2000;24(1):57–60.
358. Jain M, Arvanitis C, Chu K, Dewey W, Leonhardt E, Trinh M, et al. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science (80-). 2002;297(5578):102–4.
359. Weinstein IB, Joe A. Oncogene addiction. Cancer Res. 2008;68(9):3077–80.
360. O'Neil NJ, Bailey ML, Hieter P. Synthetic lethality and cancer. Nat Rev Genet. 2017;18(10):613–23.
361. Paul A, Paul S. The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. Front Biosci. 2014;19:605–18.
362. Li SKH, Martin A. Mismatch Repair and Colon Cancer: Mechanisms and Therapies Explored. Trends Mol Med. 2016;22(4):274–89.
363. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005;434(7035):913–7.
364. Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434(1991):917–21.
365. Leiserson MDM, Wu HT, Vandin F, Raphael BJ. CoMEt: A statistical approach to identify combinations of mutually exclusive alterations in cancer. Genome Biol. 2015;16(1):1–20.
366. Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, et al. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. Cell. 2015;160(6):1246–60.
367. Wang T, Yu H, Hughes NW, Liu B, Kendirli A, Klein K, et al. Gene Essentiality Profiling Reveals Gene Networks and Synthetic Lethal Interactions with Oncogenic Ras. Cell. 2017;168(5):890-903.e15.
368. Behan FM, Iorio F, Picco G, Gonçalves E, Beaver CM, Migliardi G, et al. Prioritization of cancer therapeutic targets using CRISPR–Cas9 screens. Nature. 2019;568(7753):511–6.
369. Peters GJ, Van Der Wilt CL, Van Moorsel CJA, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther. 2000;87(2–3):227–53.
370. Zhang Y, Hunter T. Roles of Chk1 in cell biology and cancer therapy. Int J Cancer. 2014;134(5):1013–23.
371. Krajewska M, Fehrmann RSN, Schoonen PM, Labib S, De Vries EGE, Franke L, et al. ATR inhibition preferentially targets homologous recombination-deficient tumor cells. Oncogene. 2015;34(26):3474–81.
372. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, et al. Conservation of the Chk1 Checkpoint Pathway in Mammals : Linkage of DNA Damage to Cdk Regulation Through Cdc25 Conservation of the Chk1 Checkpoint Pathway in Mammals : Linkage of DNA Damage to Cdk Regulation Through Cdc25. Science (80-). 1997;277:1497–502.
373. Peng C-Y, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science. 1997;277(5331):1501–6.
374. Mailand N, Falck J, Lukas C, Syljuåsen RG, Welcker M, Bartek J, et al. Rapid Destruction of Human Cdc25A in Response to DNA Damage. Science (80-). 2000;288:1425–30.
375. Tercero JA, Diffley JF. Regulation of DNA replication fork progression through damaged DNAby the Mec1/Rad53 checkpoint. Nature. 2001;412:553–7.
376. Murga M, Bunting S, Montaña MF, Soria R, Mulero F, Cañamero M, et al. A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat Genet. 2009;41(8):891–8.
377. Ma CX, Janetka JW, Piwnica-Worms H. Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. Trends Mol Med. 2011;17(2):88–96.
378. Murga M, Campaner S, Lopez-Contreras AJ, Toledo LI, Soria R, Montaña MF, et al. Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. Nat Struct Mol Biol. 2011;18(12):1331–5.
379. Gaillard H, García-Muse T, Aguilera A. Replication stress and cancer. Nat Rev Cancer. 2015;15(5):276–80.
380. Derenzini E, Agostinelli C, Imbrogno E, Iacobucci I, Casadei B, Brighenti E, et al. Constitutive activation of the DNA damage response pathway as a novel therapeutic target in diffuse large B-cell lymphoma. Oncotarget. 2015;6(9).

381. Sarmento LM, Póvoa V, Nascimento R, Real G, Antunes I, Martins LR, et al. CHK1 overexpression in T-cell acute lymphoblastic leukemia is essential for proliferation and survival by preventing excessive replication stress. Oncogene. 2015;34(23):2978–90.
382. Morgado-Palacin I, Day A, Murga M, Lafarga V, Anton ME, Tubbs A, et al. Targeting the kinase activities of ATR and ATM exhibits antitumoral activity in mouse models of MLL -rearranged AML. Sci Signal. 2016;9(445):1–9.
383. Kwok M, Davies N, Agathanggelou A, Smith E, Oldreive C, Petermann E, et al. ATR inhibition induces synthetic lethality and overcomes chemoresistance in TP53-or ATM-defective chronic lymphocytic leukemia cells. Blood. 2016;127(5):582–95.
384. Ducker GS, Ghergurovich JM, Mainolfi N, Suri V, Jeong SK, Li SHJ, et al. Human SHMT inhibitors reveal defective glycine import as a targetable metabolic vulnerability of diffuse large B-cell lymphoma. Proc Natl Acad Sci U S A. 2017;114(43):11404–9.
385. Kotowski K, Rosik J, Machaj F, Supplitt S, Wiczew D, Jabłońska K, et al. Role of PFKFB3 and PFKFB4 in cancer: genetic basis, impact on disease development/progression, and potential as therapeutic targets. Cancers (Basel). 2021;13(4):1–29.
386. Zhu Z, Ka G, Leung K. More Than a Metabolic Enzyme : MTHFD2 as a Novel Target for Anticancer Therapy ? Front Oncol. 2020;10(April):1–9.
387. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. J Clin Invest. 2013;123(9):3685–92.
388. Hamanaka RB, Chandel NS. Targeting glucose metabolism for cancer therapy. J Exp Med. 2012;209(2):211–5.
389. Hay N. Reprogramming glucose metabolism in cancer: Can it be exploited for cancer therapy? Nat Rev Cancer. 2016;16(10):635–49.
390. Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. Oncogene. 2006;25(34):4633–46.
391. Zhao Y, Butler EB, Tan M. Targeting cellular metabolism to improve cancer therapeutics. Cell Death Dis. 2013;4(3):1–10.
392. Wick AN, Drury DR, Nakada HI, Wolfe JB. Localization of the primary metabolic block produced by 2-deoxyglucose. J Biol Chem. 1957;224(2):963–9.
393. Zhang D, Li J, Wang F, Hu J, Wang S, Sun Y. 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. Cancer Lett. 2014;355(2):176–83.
394. Landau BR, Laszlo J, Stengle J, Burk D. Certain metabolic and pharmacologic effects in cancer patients given infusion of 2-deoxy-D-glucose. J Natl Cancer Inst. 1958;21(3):485–94.
395. Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, et al. A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors. Cancer Chemother Pharmacol. 2013;71(2):523–30.
396. Stein M, Lin H, Jeyamohan C, Dvorzhinski D, Gounder M, Bray K, et al. Targeting tumor metabolism with 2-deoxyglucose in patients with castrate-resistant prostate cancer and advanced malignancies. Prostate. 2010;70(13):1388–94.
397. Sarkar Bhattacharya S, Thirusangu P, Jin L, Roy D, Jung D, Xiao Y, et al. PFKFB3 inhibition reprograms malignant pleural mesothelioma to nutrient stress-induced macropinocytosis and ER stress as independent binary adaptive responses. Cell Death Dis. 2019;10(10).
398. Boyd S, Brookfield JL, Critchlow SE, Cumming IA, Curtis NJ, Debreczeni J, et al. Structure-based design of potent and selective inhibitors of the metabolic kinase PFKFB3. J Med Chem. 2015;58(8):3611–25.
399. Emini Veseli B, Perrotta P, Van Wielendaele P, Lambeir AM, Abdali A, Bellosta S, et al. Small molecule 3PO inhibits glycolysis but does not bind to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3). FEBS Lett. 2020;594(18):3067–75.
400. Burmistrova O, Olias-Arjona A, Lapresa R, Jimenez-Blasco D, Eremeeva T, Shishov D, et al. Targeting PFKFB3 alleviates cerebral ischemia-reperfusion injury in mice. Sci Rep. 2019;9(1):1–13.
401. Tedeschi PM, Vazquez A, Kerrigan JE, Bertino JR. Mitochondrial Methylenetetrahydrofolate Dehydrogenase (MTHFD2) Overexpression Is Associated with Tumor Cell Proliferation and Is a Novel Target for Drug Development. Mol Cancer Res. 2015;13(10):1361–6.
402. Lukey MJ, Katt WP, Cerione RA. Targeting amino acid metabolism for cancer therapy. Drug Discov Today. 2017;22(5):796–804.
403. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. Nature. 2010;463(7283):899–905.
404. DeNicola GM, Chen PH, Mullarky E, Sudderth JA, Hu Z, Wu D, et al. NRF2 regulates serine biosynthesis in non-small cell lung cancer. Nat Genet. 2015;47(12):1475–81.
405. Samanta D, Park Y, Andrabi SA, Shelton LM, Gilkes DM, Semenza GL. PHGDH expression is required for mitochondrial redox homeostasis, breast cancer stem cell maintenance, and lung metastasis. Cancer Res. 2016;76(15):4430–42.
406. Mullarky E, Lucki NC, Zavareh RB, Anglin JL, Gomes AP, Nicolay BN, et al. Identification of a small molecule inhibitor of 3-phosphoglycerate dehydrogenase to target serine biosynthesis in cancers. Proc Natl Acad Sci U S A. 2016;113(11):E1585.
407. Pacold ME, Brimacombe KR, Chan SH, Rohde JM, Lewis CA, Swier LJYM, et al. A PHGDH inhibitor reveals coordination of serine synthesis and one-carbon unit fate. Nat Chem Biol. 2016;12(6):452–8.
408. Wang Q, Liberti M V., Liu P, Deng X, Liu Y, Locasale JW, et al. Rational Design of Selective Allosteric Inhibitors of PHGDH and Serine Synthesis with Anti-tumor Activity. Cell Chem Biol. 2017;24(1):55–65.
409. Furuya S. An essential role for de novo biosynthesis of L-serine in CNS development. Asia Pac J Clin Nutr. 2008;17:312–5.
410. Yoshida K, Furuya S, Osuka S, Mitoma J, Shinoda Y, Watanabe M, et al. Targeted Disruption of the Mouse 3-Phosphoglycerate Dehydrogenase Gene Causes Severe Neurodevelopmental Defects and Results in Embryonic Lethality. J Biol Chem. 2004;279(5):3573–7.

411. Schmidt A, Wu H, Mackenzie RE, Chen VJ, Bewly JR, Ray JE, et al. Structures of three inhibitor complexes provide insight into the reaction mechanism of the human methylenetetrahydrofolate dehydrogenase/cyclohydrolase. Biochemistry. 2000;39(21):6325–35.
412. Gustafsson R, Jemth AS, Gustafsson NMS, Farnegardh K, Loseva O, Wiita E, et al. Crystal structure of the emerging cancer target MTHFD2 in complex with a substrate-based inhibitor. Cancer Res. 2017;77(4):937–48.
413. Intlekofer AM, Shih AH, Wang B, Nazir A, Rustenburg AS, Albanese SK, et al. Acquired resistance to IDH inhibition through trans or cis dimer-interface mutations. Nature. 2018;559(7712):125–9.
414. Shi L, Pan H, Liu Z, Xie J, Han W. Roles of PFKFB3 in cancer. Signal Transduct Target Ther. 2017;2(April):1–10.
415. Marston E, Weston V, Jesson J, Maina E, McConville C, Agathanggelou A, et al. Stratification of pediatric ALL by in vitro cellular responses to DNA double-strand breaks provides insight into the molecular mechanisms underlying clinical response. Blood. 2009;113(1):117–26.
416. Dart ML, Machleidt T, Jost E, Schwinn MK, Robers MB, Shi C, et al. Homogeneous Assay for Target Engagement Utilizing Bioluminescent Thermal Shift. ACS Med Chem Lett. 2018;9(6):546–51.
417. Martinez Molina D, Jafari R, Ignatushchenko M, Seki T, Larsson EA, Dan C, et al. Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal Shift Assay. Science (80-). 2013;341(6141):84–7.
418. Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundbäck T, Nordlund P, et al. The cellular thermal shift assay for evaluating drug target interactions in cells. Nat Protoc. 2014;9(9):2100–22.
419. Martinez Molina D, Nordlund P. The Cellular Thermal Shift Assay: A Novel Biophysical Assay for in Situ Drug Target Engagement and Mechanistic Biomarker Studies. Annu Rev Pharmacol Toxicol. 2016;56:141–61.
420. Schürmann M, Janning P, Ziegler S, Waldmann H. Small-Molecule Target Engagement in Cells. Cell Chem Biol. 2016;23(4):435–41.
421. Gunn A, Stark JM. I-SceI-Based Assays to Examine Distinct Repair Outcomes of Mammalian Chromosomal Double Strand Breaks. In: Bjergbæk L, editor. DNA Repair Protocols. Totowa, NJ: Humana Press; 2012. p. 379–91.
422. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev. 1999;
423. Yoshino Y, Endo S, Chen Z, Qi H, Watanabe G, Chiba N. Evaluation of site-specific homologous recombination activity of BRCA1 by direct quantitation of gene editing efficiency. Sci Rep. 2019;9(1):1–12.
424. Richardson C, Moynahan ME, Jasin M. Double-strand break repair by interchromosomal recombination: Suppression of chromosomal translocations. Genes Dev. 1998;12(24):3831–42.
425. Quinet A, Carvajal-Maldonado D, Lemacon D, Vindigni A. DNA Fiber Analysis: Mind the Gap!. 1st ed. Vol. 591, Methods in Enzymology. Elsevier Inc.; 2017. 55–82 p.
426. Nieminuszczy J, Schwab RA, Niedzwiedz W. The DNA fibre technique – tracking helicases at work. Methods. 2016;108:92–8.
427. Técher H, Koundrioukoff S, Azar D, Wilhelm T, Carignon S, Brison O, et al. Replication dynamics: Biases and robustness of DNA fiber analysis. J Mol Biol. 2013;425(23):4845–55.
428. Michalet X, Ekong R, Fougerousse F, Rousseaux S, Schurra C, Hornigold N, et al. Dynamic molecular combing: Stretching the whole human genome for high- resolution studies. Science (80-). 1997;277(5331):1518–23.
429. Daigaku Y, Davies AA, Ulrich HD. Ubiquitin-dependent DNA damage bypass is separable from genome replication. Nature. 2010;465(7300):951–5.
430. Jackson DA, Pombo A. Replicon clusters are stable units of chromosome structure: Evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. J Cell Biol. 1998;140(6):1285–95.
431. Bianco JN, Poli J, Saksouk J, Bacal J, Silva MJ, Yoshida K, et al. Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. Methods. 2012;57(2):149–57.
432. Conti C, Saccà B, John H, Lalou C, Pommier Y, Bensimon A. Replication Fork Velocities at Adjacent Replication Origins Are Coordinately Modified during DNA Replication in Human Cells. Mol Biol Cell. 2007;18:3059–67.
433. Guilbaud G, Rappailles A, Baker A, Chen CL, Arneodo A, Goldar A, et al. Evidence for sequential and increasing activation of replication origins along replication timing gradients in the human genome. PLoS Comput Biol. 2011;7(12).
434. Vindigni A, Lopes M. Combining electron microscopy with single molecule DNA fiber approaches to study DNA replication dynamics. Biophys Chem. 2017;225:3–9.
435. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun. 1984;123(1):291–8.
436. Neri M, Milazzo D, Ugolini D, Milic M, Campolongo A, Pasqualetti P, et al. Worldwide interest in the comet assay: A bibliometric study. Mutagenesis. 2015;30(1):155–63.
437. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184–91.
438. Cook PR, Brazell IA. Characterization of nuclear structures containing superhelical DNA. J Cell Sci. 1976;22:303.
439. Møller P, Loft S, Ersson C, Koppen G, Dusinska M, Collins A. On the search for an intelligible comet assay descriptor. Front Genet. 2014;5(JUL):3–6.
440. Langie SAS, Azqueta A, Collins AR. The comet assay: Past, present, and future. Front Genet. 2015;6(Aug):1–3.
441. Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. Nat Protoc. 2006;1(1):23–9.
442. Smith CC, O'Donovan MR, Martin EA. hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. Mutagenesis. 2006;21(3):185–90.
443. Duthie SJ, McMillan P. Uracil misincorporation in human DNA detected using single cell gel electrophoresis. Carcinogenesis. 1997;18(9):1709–14.
444. Dušinská M, Collins A. Detection of Oxidised Purines and UV-induced Photoproducts in DNA of Single Cells, by Inclusion of Lesion-specific Enzymes in the Comet Assay. Altern to Lab Anim. 1996 Jun 1;24(3):405–11.
445. Cortés-Gutiérrez EI, López-Fernández C, Fernández JL, Dávila-Rodríguez MI, Johnston SD, Gosálvez J. Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. Front Genet.

2014;5(NOV):1–11.
446. McAllister KA, Yasseen AA, McKerr G, Downes CS, McKelvey-Martin VJ. FISH comets show that the salvage enzyme TK1 contributes to gene-specific DNA repair. Front Genet. 2014;5(AUG):1–9.
447. Collins AR, El Yamani N, Lorenzo Y, Shaposhnikov S, Brunborg G, Azqueta A. Controlling variation in the comet assay. Front Genet. 2014;5(SEP):1–6.
448. Brunborg G, Jackson P, Shaposhnikov S, Dahl H, Azqueta A, Collins AR, et al. High throughput sample processing and automated scoring. Front Genet. 2014;5(OCT):1–6.
449. Yalcin A, Telang S, Clem B, Chesney J. Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases in cancer. Exp Mol Pathol. 2009;86(3):174–9.
450. Herr P, Lundin C, Evers B, Ebner D, Bauerschmidt C, Kingham G, et al. A genome-wide IR-induced RAD51 foci RNAi screen identifies CDC73 involved in chromatin remodeling for DNA repair. Cell Discov. 2015;
451. Adamson B, Smogorzewska A, Sigoillot FD, King RW, Elledge SJ. A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response. Nat Cell Biol. 2012;14(3):318–28.
452. Monzingo AF, Breksa A, Ernst S, Appling DR, Robertus JOND. The X-ray structure of the NAD-dependent from Saccharomyces cerevisiae. Protein Sci. 2000;1374–81.
453. Shen BW, Dyer DH, Huang J-Y, D'Ari L, Rabinowitz J, Stoddard BL. The crystal structure of a bacterial, bifunctional 5, 10 methylene-tetrahydrofolate dehydrogenase/cyclohydrolase. Protein Sci. 1999;8(6):1342–9.
454. Kingham GL. Screening for inhibitors of and novel proteins within the homologous recombination DNA repair pathway. 2012.
455. Marzano V, Santini S, Rossi C, Zucchelli M, D'Alessandro A, Marchetti C, et al. Proteomic profiling of ATM kinase proficient and deficient cell lines upon blockage of proteasome activity. J Proteomics. 2012;75(15):4632–46.
456. Schneider JG, Finck BN, Ren J, Standley KN, Takagi M, Maclean KH, et al. ATM-dependent suppression of stress signaling reduces vascular disease in metabolic syndrome. Cell Metab. 2006;4(5):377–89.
457. De Oliveira T, Goldhardt T, Edelmann M, Rogge T, Rauch K, Kyuchukov ND, et al. Effects of the novel PFKFB3 inhibitor KAN0438757 on colorectal cancer cells and its systemic toxicity evaluation in vivo. Cancers (Basel). 2021;13(5):1–24.
458. Yan S, Zhou N, Zhang D, Zhang K, Zheng W, Bao Y, et al. PFKFB3 inhibition attenuates oxaliplatin-induced autophagy and enhances its cytotoxicity in colon cancer cells. Int J Mol Sci. 2019;20(21).
459. Huang Y, Li L. DNA crosslinking damage and cancer - a tale of friend and foe. Transl Cancer Res. 2013;2(3):144.
460. Chao M, Wu H, Jin K, Li B, Wu J, Zhang G, et al. A nonrandomized cohort and a randomized study of local control of large hepatocarcinoma by targeting intratumoral lactic acidosis. Elife. 2016;5(AUGUST):1–18.
461. Wu H, Ding Z, Hu D, Sun F, Dai C, Xie J, et al. Central role of lactic acidosis in cancer cell resistance to glucose deprivation-induced cell death. J Pathol. 2012;227(2):189–99.
462. Han J, Meng Q, Xi Q, Wang H, Wu G. PFKFB3 was overexpressed in gastric cancer patients and promoted the proliferation and migration of gastric cancer cells. Cancer Biomarkers. 2017;18(3):249–56.
463. Schoors S, De Bock K, Cantelmo AR, Georgiadou M, Ghesquière B, Cauwenberghs S, et al. Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. Cell Metab. 2014;19(1):37–48.
464. Yang M, Vousden KH. Serine and one-carbon metabolism in cancer. Nat Rev Cancer. 2016;16(10):650–62.
465. Zhao H, French JB, Fang Y, Benkovic SJ. The purinosome, a multi-protein complex involved in the de novo biosynthesis of purines in humans. Chem Commun. 2013;49(40):4444–52.
466. Field MS, Kamynina E, Chon J, Stover PJ. Nuclear folate metabolism. Annu Rev Nutr. 2018;38:219–43.
467. Sirbu BM, Couch FB, Cortez D. Monitoring the spatiotemporal dynamics of proteins at replication forks and in assembled chromatin using Isolation of Proteins On Nascent DNA (iPOND). Nat Protoc. 2012;7(3):594–605.
468. Huertas P. DNA resection in eukaryotes: Deciding how to fix the break. Nat Struct Mol Biol. 2010;
469. Yue L, Pei Y, Zhong L, Yang H, Wang Y, Zhang W, et al. Mthfd2 Modulates Mitochondrial Function and DNA Repair to Maintain the Pluripotency of Mouse Stem Cells. Stem Cell Reports. 2020;
470. Hagenkort A, Paulin CBJ, Desroses M, Sarno A, Wiita E, Mortusewicz O, et al. dUTPase inhibition augments replication defects of 5-fluorouracil. Oncotarget. 2017;8(14):23713–26.
471. Yamamoto N, Hayashi H, Planchard D, Morán T, Gregorc V, Dowell J, et al. A randomized, phase 2 study of deoxyuridine triphosphatase inhibitor, TAS-114, in combination with S-1 versus S-1 alone in patients with advanced non-small-cell lung cancer. Invest New Drugs. 2020;38(5):1588–97.
472. Saito K, Nagashima H, Noguchi K, Yoshisue K, Yokogawa T, Matsushima E, et al. First-in-human, phase I dose-escalation study of single and multiple doses of a first-in-class enhancer of fluoropyrimidines, a dUTPase inhibitor (TAS-114) in healthy male volunteers. Cancer Chemother Pharmacol. 2014;73(3):577–83.
473. Miyahara S, Miyakoshi H, Yokogawa T, Chong KT, Taguchi J, Muto T, et al. Discovery of a novel class of potent human deoxyuridine triphosphatase inhibitors remarkably enhancing the antitumor activity of thymidylate synthase inhibitors. J Med Chem. 2012;55(7):2970–80.
474. Thomas A, Redon CE, Sciuto L, Padiernos E, Ji J, Lee MJ, et al. Phase I study of ATR inhibitor M6620 in combination with topotecan in patients with advanced solid tumors. J Clin Oncol. 2018;36(16):1594–602.
475. Shukla K, Singh N, Lewis JE, Tsang AW, Boothman DA, Kemp ML, et al. MTHFD2 Blockade Enhances the Efficacy of β-Lapachone Chemotherapy With Ionizing Radiation in Head and Neck Squamous Cell Cancer. Front Oncol. 2020;10(November):1–12.
476. Wan X, Wang C, Huang Z, Zhou D, Xiang S, Qi Q, et al. Cisplatin inhibits SIRT3-deacetylation MTHFD2 to disturb cellular redox balance in colorectal cancer cell. Cell Death Dis. 2020;11(8).
477. Shang M, Yang H, Yang R, Chen T, Fu Y, Li Y, et al. The folate cycle enzyme MTHFD2 induces cancer immune evasion through PD-L1 up-regulation. Nat Commun. 2021;12(1):1940.
478. Matherly LH, Hou Z, Deng Y. Human reduced folate carrier: Translation of basic biology to cancer etiology and therapy. Cancer Metastasis Rev. 2007;26(1):111–28.
479. Zhao R, Goldman ID. The molecular identity and characterization of a Proton-Coupled Folate Transporter - PCFT;

biological ramifications and impact on the activity of pemetrexed-12 06 06. Cancer Metastasis Rev. 2007;26(1):129.
480. Elnakat H, Ratnam M. Distribution, functionality and gene regulation of folate receptor isoforms: Implications in targeted therapy. Adv Drug Deliv Rev. 2004;56(8):1067–84.
481. Lawrence SA, Hackett JC, Moran RG. Tetrahydrofolate recognition by the mitochondrial folate transporter. J Biol Chem. 2011;286(36):31480–9.
482. O'Connor C, Wallace-Povirk A, Ning C, Frühauf J, Tong N, Gangjee A, et al. Folate transporter dynamics and therapy with classic and tumor-targeted antifolates. Sci Rep. 2021;11(1):1–14.
483. Galivan J, Ryan TJ, Chave K, Rhee M, Yao R, Yin D. Glutamyl hydrolase: pharmacological role and enzymatic characterization. Pharmacol Ther. 2000;85(3):207–15.
484. Maezawa Y, Sakamaki K, Oue N, Kimura Y, Hashimoto I, Hara K, et al. High gamma-glutamyl hydrolase and low folylpolyglutamate synthetase expression as prognostic biomarkers in patients with locally advanced gastric cancer who were administrated postoperative adjuvant chemotherapy with S-1. J Cancer Res Clin Oncol. 2020;146(1):75.
485. Smit EF, Socinski MA, Mullaney BP, Myrand SP, Scagliotti G V., Lorigan P, et al. Biomarker analysis in a phase III study of pemetrexed-carboplatin versus etoposide-carboplatin in chemonaive patients with extensive-stage small-cell lung cancer. Ann Oncol. 2012;23(7):1723–9.
486. Wang X, Yin L, Rao P, Stein R, Harsch KM, Lee Z, et al. Targeted treatment of prostate cancer. J Cell Biochem. 2007;102(3):571–9.
487. Leamon CP, Reddy JA, Dorton R, Bloomfield A, Emsweller K, Parker N, et al. Impact of High and Low Folate Diets on Tissue Folate Receptor Levels and Antitumor Responses Toward Folate-Drug Conjugates. J Pharmacol Exp Ther. 2008;327(3):918–25.
488. Blom K, Nygren P, Larsson R, Andersson CR. Predictive Value of Ex Vivo Chemosensitivity Assays for Individualized Cancer Chemotherapy: A Meta-Analysis. SLAS Technol. 2017;22(3):306–14.